Vol. 25, No. 3, November-December, 1960
Russian Original Dated May-June, 1960

BIOCHEMISTRY

БИОХИМИЯ

BIOKHIMIYA

translated from Russian

CONSULTANTS BUREAU

THE LATEST SOVIET RESEARCH — IN TRANSLATION

BULLETIN OF EXPERIMENTAL BIOLOGY AND MEDICINE

Features research reports by leading Soviet authorities on *physiology, pathology, immunology, biophysics, pharmacology, oncology, morphology* and related fields. Translation is by special arrangement with the National Institutes of Health.

ANNUAL SUBSCRIPTION: 12 issues, **$20.00.**

BACK ISSUES: 1956-58 (12 issues per year), **$20.00** per year.

BIOCHEMISTRY

The most comprehensive Soviet periodical in this field. Each issue contains more than twenty papers on the biochemistry of plants and animals. Translation is by special arrangement with the National Institutes of Health.

ANNUAL SUBSCRIPTION: 6 issues, **$20.00.**

BACK ISSUES: 1956-58 (6 issues per year), **$20.00** per year.

PHARMACOLOGY AND TOXICOLOGY

This journal contains reports of the latest Soviet research on physiological effects of drugs, industrial hygiene, and related chemico-pharmacological-toxicological fields. The translation is published by special arrangement with the American pharmaceutical industry.

ANNUAL SUBSCRIPTION: 6 issues, **$25.00.**

BACK ISSUES: 1957-58 (6 issues per year), **$25.00** per year.

ANTIBIOTICS

This outstanding new publication surveys the entire field with timely research reports by leading Soviet scientists. It is translated and published with the financial assistance of a number of leading U.S. companies.

ANNUAL SUBSCRIPTION: 6 issues, **$25.00.**

Descriptive folders upon request

and—of special interest—

SOVIET RESEARCH IN PHARMACEUTICAL CHEMISTRY

Two invaluable collections which provide researchers with a convenient and inexpensive coverage of contemporary advances in Soviet pharmaceutical research.

Contents include pertinent articles which have appeared in chemistry journals translated cover-to-cover since 1949. Presents Western scientists with an excellent insight into the thinking, methodology and trends of their Soviet counterparts.

"a storehouse of factual references" —Journal of Chemical Education

Tables of contents and prices upon request.

CONSULTANTS BUREAU

227 West 17th Street, New York 11, N.Y.

EDITORIAL BOARD OF BIOKHIMIYA

V. A. Engel'gardt *(Editor)*
N. M. Sisakyan *(Assistant Editor)*
A. L. Kursanov
A. I. Oparin
A. V. Palladin

BIOCHEMISTRY

A translation of BIOKHIMIYA, a publication of the Academy of Sciences of the USSR

Russian Original Dated May-June, 1960

Vol. 25, No. 3, pp. 297-442 November-December, 1960

CONTENTS

	PAGE	RUSS. PAGE
Basic Problems of Radiation Biochemistry. A. G. Pasynskii and N. N. Demin.	297	385
The Preparative-Scale Isolation of Cytidylic Acid by Ion Exchange. L. A. Filippova and M. A. Prokof'ev	303	393
The Rate of Cholesterol and Fatty Acid Synthesis in the Adrenals, Testes, and Liver of Young and Old Rats, Both Normal and Irradiated. K. A. Tret'yakova and D. É. Grodzenskii.	307	399
The Transformation of Flavonoids in Tea Production. M. A. Bokuchava and N. I. Skobeleva	311	404
The Regulatory Significance of the DPN^+–DPNH System in Intermediate Carbohydrate Metabolism. A. I. Kolotilova.	313	407
A Comparative Immunological Investigation of Nucleoproteins Isolated from Regenerated Filterable Forms of Typhoid Fever Bacteria. A. N. Bitkova and E. I. Koptelova.	320	417
The Question of the Binding of Labeled Methionine-S^{35} by Proteins. M. S. Volkova, L. V. Komarova, and A. G. Pasynskii	324	422
Biochemical Changes in the Organism of the Carp Produced by Administration of Antigens. Ya. A. Epshtein, B. G. Avtikyan, N. E. Lavrovskaya, V. M. Rogozhnikova, and A. G. Artemova	328	427
On the Properties and Intracellular Distribution of the L- and D-Amino Acid Oxidases in the Kidney. A. V. Azyavchik	335	436
The Binding of Labeled Amino Acids by Protein and Nucleic Acid Preparations. O. P. Samarina, I. B. Zbarskii, and K. A. Perevoshchikova	339	443
Variability of P/O Ratios in Oxidative Processes. V. P. Skulachev and L. L. Kiselev	346	452
The Absorption by Fish of Inorganic Carbon from the Surrounding Media, and its Participation in Metabolism. K. F. Sorvachev and O. V. Belokopytova	351	459
Ascorbic Acid Metabolism in the Walls of the Aorta in Development of Radiation Injury. L. I. Polikarpova	356	465
Adenylic Acid System in Tissue Culture Cells under Aerobic and Anaerobic Conditions. V. I. Agol and I. V. Skarlat	359	470
The Assimilation of Labeled Ammonia from the Soil by the Root System. V. L. Kretovich, Z. G. Evstigneeva, and K. B. Aseeva	363	476
The Cytochrome System in the Cytoplasmic Membranes of Micrococcus lysodeikticus. N. S. Gel'man, M. A. Lukoyanova, and A. I. Oparin	368	482
A Study of Phosphorus Metabolism of the Green Photosynthesizing Sulfur Bacterium in Relation to Carbon Dioxide Fixation. V. H. Shaposhnikov and V. D. Fedorov	372	487
The Oxidative Function of Peroxidase from Cabbage (Brassica oleracea L.). T. M. Ivanova and B. A. Rubin	379	496
Changes in Some Properties of Myosin, Actomyosin, and Actin under the Influence of High Pressure. I. I. Ivanov, Yu. N. Bert, and N. A. Lebedeva.	386	505

Annual Subscription $20.00
Single Issue 10.00

© 1960 Consultants Bureau Enterprises, Inc., 227 West 17th St., New York 11, N.Y.
Note: The sale of photostatic copies of any portion of this copyright translation is expressly prohibited by the copyright owners.

CONTENTS (continued)

	PAGE	RUSS. PAGE
Changes during Storage in the Chemical Composition of a Variant of Brucella Obtained under the Influence of Phage. I. I. Dubrovskaya and N. N. Ostrovskaya	390	511
Nucleoprotein Fractions of Vaccine Strains of Plague Bacteria. M. L. Bekker, A. Z. Kutsemakina, and E. A. Chernova	396	517
Pentose Metabolism and the Effect of Orthophosphate on the Path of Degradation of Sugars in Streptomyces aureofaciens. Shên Sang-ch'ung and Ch'ang Yung-P'u	401	523
The Amphoteric Properties of the Actin-Like Protein from the Smooth Muscle of Dog Stomach. V. V. Oppel' and T. B. Khlyustina	409	532
The Effect of Ionizing Radiation on the Phospholipid Metabolism in the Liver. A. S. Kainova	415	540
The Biosynthesis of Higher Acids from Acetate in Isolated Chloroplasts of Spinacia oleracea Leaves. B. P. Smirnov	419	545
The Effect of Insulin on Glycolysis in Tissue Culture Cells. R. N. Étingof and A. A. Krichevskaya	427	556
The Interaction of Purine and Pyrimidine Derivatives with Formaldehyde in Aqueous Solutions. M. Ya. Fel'dman	432	563
An Apparatus for Automatic Placing of Spots on Chromatographic Paper. M. M. Zaalishvili and F. O. Shraibman	438	570
Congress Tribune	440	573

The National Institutes of Health of the Public Health Service has supported in part the cost of publishing BIOCHEMISTRY through the purchase of copies for distribution to American medical libraries.

BASIC PROBLEMS OF RADIATION BIOCHEMISTRY

A.G. Pasynskii and N.N. Demin

Translated from Biokhimiya Vol. 25, No. 3, pp. 385-392,
May-June, 1960

The great problems of building communism in our country open up broad perspectives for the peaceful use of atomic energy in all branches of the national economy, medicine and scientific research: Progress in the production of atomic energy, the increase in the number of atomic stations, of various nuclear reactors and of atomic particle accelerators, the widespread use of radioactive isotopes as sources of radiation and as indicators, all are unavoidable accompanied by a considerable increase in the number of persons who come into contact with nuclear radiations. The waste of atomic production also can have an effect on animals, plants and microorganisms, on the world of living substances on land, in the water, and in the air. The prevention of the harmful effects of radiation is one of the most important problems of cosmic flight. Ionizing radiation is becoming at the present time a new, important factor of outer space which exerts a substantial influence on all living organisms (and, in patricular, on the human organism) which have not undergone evolutionary adaptation to this influence. As a result, the problem of the biological effect of ionizing radiation is one of the leading problems of contemporary science. On its solution will depend not only the development of biology and medicine, but also in a series of instances the direction of the technical utilization of atomic energy.

A profound knowledge of the mechanism of the biological effect of radiation is necessary for the effective protection of living organisms from the harmful influence of radiation, and equally for the efficient use of the effect of radiation on living organisms. This knowledge can be based only upon clarification of the biochemical principles of radiobiological effect, on the study of the changes in metabolic processes in the living organism caused by the action of radiation. In this respect investigations in the field of radiation biochemistry undoubtedly have a basic value for all radiation biology.

It must be acknowledged that questions of the biological effect of ionizing radiation present biochemistry with exceptionally complicated and difficult problems. In essence, all the problems of biochemistry -- questions of albuminous, nucleinic, carbohydrate and lipid change, of the structure of molecules of all forms of biopolymers, a majority of the problems of general, medicinal and technical biochemistry must be investigated with regard to the distrubances and changes brought about by the influence of radiation, since all of them are altered to some extent and acquire new and original features which must be the subject of special study. Thus it is necessary to work through all of biochemistry, as it were anew, from the aspect of radiation. A series of specific problems confronts radiation biochemistry, questions which have fundamental importance for this area of biochemistry and distringuish it from the broader fields of radiation biology which include many other problems.

The fact is, that although ionizing radiation appears to be a powerful physical factor with an effect on living organisms, at the very moment of this effect it causes, in the substances of living organisms, chemical changes, which appear to be the cause of all the subsequent biochemical, physiological, and morphological distrubances. Thus, at the initial moments of the effect of radiation on living organisms, the physical, chemical and biological factors are closely intermingled with one another; consequently, radiation biochemistry as a branch of science is closely connected with such disciplines as the physics of the interaction of radiation with a substance and radiation chemistry. Therefore, physical problems are important to radiation chemistry; such problems as the mechanism of the transfer of energy from ionizing particles, the spatial distribution of ionization, the formation of radicals, of new and excited molecules in an exposed substance, the yield of these products, their energy level, the kinetics of the reaction between them and other particles, their deactivation or preservation, the effect of the magnitude of the dose and of different types of radiation, the effect of the dimensions and state of aggregation of the molecules of the medium, etc.

Of still greater importance for radiation biochemistry are investigations in the radiation chemistry of water, of aqueous solutions and of simple and complex organic substances. The nature and the of radiolysis of water under different conditions of radiation exert an important influence on the rate of a series of biochemical processes. The reactions caused by the action of radiation—deamidization, oxidation, decarboxylation, isomerization, destruction or, conversely, polymerization, the formation of chemical bonds ("seams") between molecules—are matters of great interest for radiation biochemistry, which grows still more when one considers the substances of biochemical importance—amino acids, simple carbohydrates, thiols, fatty saturated and unsaturated acids, phospholipids, purines and pyrimidines, mononucleosides and nucleotides, and others. Apart

from the chemical changes which have been mentioned, in the action of radiation on pure substances and their aqueous solutions, the study of dependence of chemical radiation yields upon the size and strength of the dose, the form of radiation, temperature and composition of the medium, the dimensions and structure (linear, cyclic, branched) of the molecule, etc., is of great importance. In mixtures of various compounds even at this level an action that is both shielding and sensitizing can be observed, depending, for example, on the correlation of the ionization potentials and the excitation of the compounds, on the correlation of the compounds and other factors; the importance of establishing quantitative rules in this area appears sufficiently obvious.

It is also meaningful for radiation biochemistry that radiational chemical changes in complex molecules of polymers often differ noticeably from analogous changes in the corresponding lower-molecular substance, because in complex molecules the probability of internal conversion rises. It is likewise necessary to study disturbances of the regularity of a macromolecular structure originating in local chemical changes and capable of extending, for example, to tens of atomic radii from the place of a given "seam".

Inseparable parts of radiation chemistry proper are investigations of radiation chemical changes in pure albumins, enzymes, hormones, nucleic acids, polysaccharides, various proteins, etc. Complete knowledge must be obtained of changes in the chemical makeup of alumins—the oxidation of mercapto groups, chromophoric groupings, disturbances of spatial structure, the combining capacity of various organic substances, the study of radicals and excited states in albumin molecules, radiation aftereffects in albumins. In studying the effects of exposure of biologically active albumins (enzymes, albuminous hormones, etc.), side by side with the clarification of all these problems, the comparison of chemical and structural changes with changes of specific activity and the determination of the dimensions and structure of active centers (see below), and the like, have great importance. With regard to nucleic acids, in addition to study of chemical changes, the process of polymerization is of special interest, as are changes in resistance to hydrolysis and in the dimensions and hydrodynamic properties of the molecules; for biologically active preparations (transforming DNK, virus nucleic acids), the study of the dependence between radiation damage and changes in the transmission of information, even in other forms of specific activity, is of great interest. With regard to pure lipids there is much interest in the investigation of oxidation of unsaturated fatty acids, the formation by them of peroxides and aldehydes and the study of their chemical nature and toxic properties. No less important is the study of the effect of radiation on various proteins—nucleoproteins, lipoproteins, glycoproteins, chromoproteins—and also on albuminous complexes, including complexes of albumins with one another, complexes of enzymes with other substances, etc., since the decomposition of these proteins or complexes, or the screening effect of one of the components of a complex on another component in the presence of radiation, has great biochemical interest.

Another of the interesting directions of investigation is the application of the statistical theory of radiation inactivation to determine the dimensions of biologically active molecules or active centers of the molecules of enzymes, hormones, viruses, alkalides and others in the pure form or even in the form of impure preparations (the latter case has special importance, as these substances are sometimes difficult to prepare in the high purity or crystalline state necessary for the determination of their dimensions by ordinary methods). By combining exposure to protons or deuterons with exposure to electrons, it is possible in this way to arrive at a determination not only of the dimensions but also of the configuration of the investigated molecules.

At this level of research on the effect of radiation on the basic classes of biopolymers and biochemically important substances a number of important problems arise which have a general significance for all subsequent biochemical investigations but partially also refer to simpler substances.

To avoid repetition in the future we will point out that among such general problems which embrace all levels of the effect of radiation—from modifications of albumin molecules to the rupture of chromosomes and general disease of the organism—are, for example, problems of the direct and indirect effect of radiation, of shielding or synergetic effect of present or added substances, the relative roles of the various radiolysis products of water (H, OH, HO_2, H_2O_2 and others), organic radicals and excited molecules, the peroxides and aldehydes of organic molecules, the role of the oxygen effect (which appears to be one of the fundamental effects in radiobiology), of radiation aftereffect, the influence of the temperature, composition and concentration of the solution, migration of energy of excitation, and many others. All these problems must be investigated in the presence of the effect of various forms of radiation—x-rays and gamma-rays, proton, deuteron, electron and neutron beams at various magnitudes of the ionizing particles and intensities of their current, and likewise in the presence of the effect of various radiomimetic substances. This program for the various levels of biochemical investigations still remains hardly started. It is also necessary to note these investigations must lead to the development of a quantitative theory and the establishment of a series of precise quantitative rules, which in radiation chemistry are still very small in number.

But all the directions of investigation enumerated above represent only the foundation of radiation biochemistry in which it is especially closely united with radiation chemistry. For radiation biochemistry proper, substantial interest is presented not only by chemical or structural modifications of the molecules of biopolymers or other biologically important substances. In the living cell they do not act in a homogeneous solution, but often enter into the composition of complex intracellular structures, of the nucleus, mitochondria, microsomes, plastids, and various membranes and protoplasmic surfaces of separation. Injury of the molecule in a homogeneous solution and injury of the same molecule in one of the intracellular membranes, for example, in a bimolecular layer of lipids, can have a completely different aftereffect on the activity of the cell. Chemical changes, the destruction and "seaming" of injured molecules located in thin surfaces of separation, can lead to a sharp modification of permeability or of the surface potentials, with subsequent disturbances of the normal course of the processes of exchange. The study of change in the properties of biopolymeric molecules in a film or monomolecular state makes up an important class of the investigations of radiation biochemistry, and one which still is poorly developed. In view of the importance of investigations of chemical and structural modifications in exposed molecules of biopolymers and other biologically important substances, it should be noted that in these investigations the role of the intracellular structures and the complex heterogeneity of the internal structure of the cytoplasm are often ignored. The primary mechanisms of the effect of radiation are sometimes discussed in the literature as if the cell were simply a homogeneous solution of various substances, while the complex intracellular structures form qualitative features in the effect of radiation on a living cell in comparison with their effect on isolated substances of the cell and divide radiation biochemistry and radiobiology proper from the radiation chemistry of complex molecules.

In the action of radiation upon a living cell there occurs not only an immediate modificationof the chemical composition or quantitative content of various substances, but likewise a disturbance of the spatial coordination of the processes of exchange. Any cell of a living body functions in conditions of constant diffusion of metabolites in the cell and the transfer of reaction products from the cell into the environment, that is, forms a so-called open system. It is natural therefore that a theory of the action of radiation on living organisms must take into consideration the total properties of the chemical processes in open systems. It is essential to note that for stationary concentrations of components in open systems the permeability or diffusion constants have no less an essential value than the coefficients of the rate of chemical reaction (of enzymatic processes, for example). Calculation of transfer coefficients on an equal basis with the reaction rate coefficients in the theory of open systems reveals a continuous connection of the spatial and temporal organization of the processes of exchange of substances. The effect of radiation on open systems can in general be formulated as a disturbance of the stationary state of the system which, within given limits, is compensated for by an open system and, in the presence of more intensive effects, leads to the impossibility of establishing a new stationary state and to degradation of the system. In this circumstance an essential feature of the biological effect of radiations is their ability to cause significant physiological modifications in the presence of the effects of very small total energies, affecting at first only very small portions solely with molecular dimensions.

Thus, one of the fundamental problems of radiation biochemistry is explaining how the injury of only a few narrowly localized portions of molecular dimensions can lead to death of the cell, since the injury of hundreds of other analogous molecules cannot exhibit a lethal effect; at the same time, this problem is closely connected with the explanation of the mechanism of "biological strengthening" of the primary radiational effects.

The investigation of this fundamental problem should be developed in various directions. First of all, attention is attracted by the study of the role of injury to unique structures of the cell, to similar chromosomes (the dependence of the number of aberrations on the intensity of the dose, the temperature, the form of radiation etc., and corresponding disturbances of biosynthetic processes), especially in connection with the genetic aftereffects of radiation. On a level with this is the great interest in the study of enzymatic reaction chains, since even a slight injury of molecules of enzymes and substrates (e.g., 5-10%) in each link of the conversions can have considerable effect on the final stages of the reactions, as has been demonstrated in a series of model systems.

A definitely important significance should be attributed to disturbance of biochemical processes (tissue respiration, oxidizing phosphorylization, the syntheses of albumins, etc.) as a result of radiational inactivation of individual structural links of mitochondria, microsomes, etc. Interest is also offered by the study of the development of chain reactions in a biosubstrate (in the lipid phase, for example) in the presence of radiation. Finally, there must be substantial significance in the injury of molecules in thin and monomolecular boundaries of a segment within a cell, leading to disturbance of the normal spatial organization of metabolic reactions, for example, the disturbance of the interaction conditions of enzymes and substrates when the enzymes can obtain access to portions of the cytoplasm from which they normally are excluded, etc. This fact can be the source of

appreciable disturbance in the course of the processes of exchange of the modified course of biosynthesis, of the formation of anomalous substances even in cases where the molecules of the enzymes and substrates themselves remain little modified under the effect of radiation. It is also necessary to have in mind that during the irradiation of cellular structures a shift can occur in the sorption equilibrium of the enzymes, the transition of part of the linked ferments into solution or the splitting-off of part of the natural inhibitors with a corresponding shift of the stationary state of the processes of assimilation–dissimilation in the cell.

A detailed study and explanation is necessary of the relative roles of all the enumerated mechanisms which evidently are also the basis of disturbances, developing in the cell, of the exchange processes of substances, gradually embracing more and more all the forms of this exchange–albuminous, nuclein, carbohydrate, lipid and others. The different radiation sensitivity of various enzymes of the systems aggravates the disturbance of the normal coordination of the exchange processes, and this is also an important direction of research. In particular, it is necessary to explain clearly in albumin exchange the change in the rate of inclusion of various amino acids, disturbance of the processes of biosynthesis of albumins, change of antigenic and other substances of serous albumins (in this connection the question of so-called C-reactive albumin and the properdin system in radiation sickness). There is substantial significance in nuclein exchange in the study of the change in the nucleotide composition of nucleic acids, especially of the high radiosensitivity of nuclein exchange, of the change in free nucleotide content, the exchange of phosphorus radicals, etc. In the metabolism of lipids and, in particular, of the phospholipids, the accumulation of peroxides and aldehydes of unsaturated fatty acids presents great interest, as does the study of their toxic properties and of the change of ratio of the various fatty acids in the formation of lipids. In the metabolism of the carbohydrates attention is attracted by the modification of specific saccharides, glycoproteins, the glycogen content in tissues, etc. It is necessary also to conduct investigations on the study of water and mineral exchange in an exposed organism, in particular the study of the metabolism of microelements and of exchange in bone tissue, etc.

It is of interest that in the living organism a depression of the various forms of exchange often occurs through an intermediate stage of activation or strengthening of this or another form of this enzymatic conversion; the study of this circumstance, following from the theory of open systems, also has great practical significance.

The briefly enumerated disturbances of the various types of exchange of substances constitute the biochemical basis of radiation sickness. A complete enumeration of the possible forms of these disturbances does not seem possible, but it is expedient to divide research in the area of radiation biochemistry into three sections: 1) Research on insulating substances or enzyme systems (including the usual biochemical experiments with extracts or homogenates of tissues), which were considered in detail above; 2) investigations of the cellular or tissue level and 3) investigations of complete multicellular organisms.

The important meaning of investigations on the cellular level, using methods of modern ultrastructural cytology, cytochemistry and cytophysiology, are obvious enough. Radiational disturbances of exchange processes ought to be studied in the various intra-cellular structures (in the nucleus, mitochondria, microsomes, etc.), and in the cytoplasm of cells of various types, of differing degrees of specialization and differentiation, the topology of biochemical changes should be studied in the cell; the biochemical bases of different radiosensitivity of various tissues and the increased sensitivity of rapidly acting cells should be explained, as well as questions of the reactivation and repair of damaged cells, the role of the shift of oxidation-reduction conditions in cells during exposure, etc. Organization of compatible cyto- or histochemical and biochemical investigations with strict quantitative calculation of the observed changes is necessary. In a more detached perspective, vast importance can attach to the directed study of the chemical composition and structure of chromosomes and cytoplasmatic structures during the action of radiation with precise determination of the broad beam, the large and intensive dose, the physiological condition of the cells and other conditions of exposure.

The growth of radiation sickness in a many-celled organism is, however, the result not only of local damage to a given group of molecules or cells by ionizing radiation, but due to reflex, trophic and humoral influences conditioned, on the one hand, by the effect of products of pathological metabolism and on the other by compensatory and reparative reactions of the organism. Although the primary biochemical injuries are, in essence, the basis of all the pathological and pathomorphological developments of the radiation damage, the biochemistry of radiation sickness in higher organisms, especially in man and animals, is considerably more complicated than in simpler organisms.

Little time has as yet been spent on studies of the biochemical basis of early damages to the nervous system and nervous control in its functional and trophic aspects, to injury to the metabolism of the series of neuromediators (acetylcholine, γ-amino acids, serotonin, etc.) and evaluation of the role of these injuries in the pathogenesis of radiation sickness, as well as injuries to endocrine systems (in

particular the hypophysial–adrenal system, the metabolism of the cortico-steroids, etc.) during radiation injury. It is necessary to study in detail injuries to the metabolism of individual vitamins in tissues, the causes and pathophysiological significance of these injuries, and likewise to solve the question of the dietetic value of individual vitamins for the prophylaxis and therapy of the various forms of radiation sickness. Biochemists should assist dietologists in the solution of the series of problems which arise in developing prophylactic and medical food rations in radiation injury. It is extremely important to study the biochemical bases of impairment of blood formation and of the hemorrhagic syndrome and to explain the conditions for the restoration of normal blood formation. The study of changes in immune substances of organisms during exposure and increased tolerance of exposed animals to "foreign" albumins or bone marrow opens up broad perspectives. No less importance attaches to the study of the biochemical principles of the carcinogenic effect of ionizing radiation or, under other conditions, the contrasting use of these radiations in the struggle against cancer. It is also necessary to work out practicable methods of early diagnosis of radiation sickness, especially in the case of chronic effects of ionizing radiation in small doses and in the determination of so-called "permissible" doses. In particular, interest is presented in this connection by work on the study of albuminous fractions of serum and changes in their antigenic properties and by data on the increased secretion of desoxycytidine, etc. The problem of remote aftereffects of radiation sickness also introduces a series of biochemical problems. Directly connected to this problem is the study of the regularity of effect of radiation on heredity, in particular an explanation of the hereditary damages to man during exposure and a search for biochemical ways to prevent or reduce these injuries. The radiation genetics of living organisms is one of the most important areas of radiobiology, which is closely associated with problems of radiation biochemistry. It is also necessary to point out the importance of biochemical research upon the effect of radiation on various stages of embryogenesis.

The problem of shielding higher organisms from the effect of radiations and of medical treatment for radiation sickness is of special importance, although, as has been pointed out, questions of shielding should be kept in view also at the molecular and cellular levels of research. The study of the mechanics of the effect of shielding substances should help both the explanation of the pathogenesis of radiation sickness and rational searches for new, effective preparations. The investigation of the biochemical mechanism of prevention or reduction of changes in nucleic acids and albumins, the elimination of excited states, the blocking of radicals formed during the action of radiation, the prevention of chromosome aberrations, the use of various antioxidants, can have an important practical value. It is also necessary to work out the biochemical bases of the struggle with the influence of incorporated radioactive substances and their effective removal from the organism. As has already been pointed out, a very important problem is that of the raising of the immunobiological tolerance of exposed animals (as regards introduced albumins, bone marrow, etc.), which likewise opens up new perspectives in the technique of transplanting tissue and organs to such animals.

A large and important part of the research is the application of radiation biochemistry to industry and agriculture. This refers to problems of the radiational sterilization of biologically active preparations, vaccines, hormones, medical sera, transplantable tissues and food products; to the use of radiation to eliminate sprouting of potatoes or the roots of sugar beets, the irradiation of seed before planting, the production of mutants of useful microorganisms with an increased yield of antibiotics, vitamins, steroids or enzymes, or with new forms of biochemical activity, the creation of new and valuable kinds of plants, the study of the effect of small doses of radiation on the growth and increase of yielding capacity of agricultural plants, the application of radiation to change the technology of producing certain food products (wine, tea, etc.).

Thus, at the present time and in coming years biochemistry is faced with very important and responsible tasks. Radiation biochemistry is becoming one of the basic areas of biochemistry. This circumstance presents large requirements also for questions of the organization of research in radiation biochemistry. Abroad, in the first place, much attention is given to working out the questions of the biological effect of ionizing radiations in the U.S.A., England, France, Sweden, and Belgium. It is sufficient to point out that of all the funds allotted to scientific research by the Atomic Energy Commission in 1957 in the U.S.A., 40% was spent on the study of the biological effect of ionizing radiation. The organization of such investigations is of course expedited in large-scale atomic centers where the biological laboratories work closely with physical and chemical laboratories organized around up-to-date sources of radiation (atomic piles, cyclotrons, linear accelerators, etc.). On this basis it is easy to assure the application of all the newest methods of physical and chemical research to the problems of radiation biochemistry. It would also be very important to organize specialized journals devoted to questions of the biological effect of radiation.

Finally, one of the decisive links in the successful development of work on radiation biochemistry is the extensive training of cadres including biologists as well as chemists and physicists. These cadres should possess theoretical preparation assuring the possibility of their

creative contact and mutual understanding in working out complex problems of radiation biochemistry, into which the interests of a number of sciences are interwoven. At the same time they should be highly trained in their specialty, since it is clear from the scope of the basic problems in radiation biochemistry outlined above that the successful development of this important area of modern biochemistry can be assured only by the united efforts of physicists, chemists, biochemists, cytologists, geneticists, and other specialists.

THE PREPARATIVE-SCALE ISOLATION OF CYTIDYLIC ACID BY ION EXCHANGE

L. A. Filippova and M.A. Prokof'ev

M.V. Lomonosov State University, Moscow.

Translated from Biokhimiya Vol. 25, No. 3, pp. 393-398, May-June, 1960
Original article submitted July 13, 1959

The preparative isolation of pure cytidylic acid from a mixture of four nucleotides presents considerable difficulties in view of the chemical similarity of these compounds with a common structure.

The laborious chemical methods for the separation of cytidylic acid from other nucleotides and hydrolysis products have recently been replaced by the more modern procedures employing ion-exchange resins. The separation of the nucleotides is usually carried out on strongly basic anion -exchangers and is made possible by the presence of a phosphoric acid residue in the nucleotide molecule. However, in view of the fact that three of the four bases which make up RNA namely, cytidine, adenine and guanine, also contain amino groups, the corresponding nucleotide mixtures can also be separated on cation-exchange resins in the hydrogen form.

The use of cation-exchangers for the retention of nucleotides is particularly convenient in cases when these need to be separated from inorganic phosphate, which in some cases is present in large amounts in the product of acid, alkaline or enzymatic hydrolysis of biological material. Cation-exchangers are also useful in the separation of the two pyrimidine nucleotides from each other, since the passage of their mixture through a suitably prepared column results in the retention of the cytidylic acid on the column, due to the presence of an amino group in the nucleotide, while the uridylic acid, which has no amino group, emerges with the effluent. The latter principle was orginally applied by Harris and Thomas [1], who isolated and fractionated pyrimidine nucleosides from a RNA digest using the cation-exchanger ZeoKarb 215. Cytidine was retained by the sulfonic resin, while uridine, containing no basic group, passed with the effluent. Occluded uridine could be removed quantitatively from the column by washing with water, while the cytidine was later eluted with 2% pyridine.

The present work deals with the isolation of cytidylic acid from biological material using domestic ion-exchange resins. In similarity with the method described by Andreev [2] for the isolation of the hypotensive hormone, pancreas tissue was dehydrated and defatted by alcohol treatment and allowed to stand in the cold for 24 hours in 0.9% NaCl with intermittent stirring. The extract was separated from the debris and precipitated with acetone. When the acetone concentration in the solution reached 37%, the preparation containing the hypotensive principle precipitated. The remaining supernatant was found to contain large amounts of phosphorus. Investigation of this solution by paper chromatography in the solvent: isobutyric acid-ammonia-water (66:1:33) [3] revealed the presence of large amounts of inorganic phosphate together with some nucleotides, mainly cytidylic acid, with traces of adenylic acid and some unidentified phosphorus-containing compounds with an R_f of 0.09 and 0.21, respectively. The nucleotides accounted for about 25-35% of the total phosphorus present in the solution. This material was used for the isolation of the cytidylic acid.

The presence of large amounts of cytidylic acid in these solutions was probably a result of RNA-ase action on the pancreatic RNA during the isolation of the hypotensive hormone involving long periods of standing at room temperature. Ribonuclease action on RNA is known to liberate ribonucleotides which are predominantly, or sometimes exclusively, pyrimidine nucleotides. The precipitate obtained by adding three volumes of acetone to seven volumes of a 0.9% NaCl extract of pancreas, was also used as a source of cytidylic acid. The precipitate contained nucleoprotein contaminated with large quantities of protein.

EXPERIMENTAL

The Isolation of Cytidylic Acid from a Ribonuclease Hydrolysate of Pancreatic RNA.

The 37% acetone supernatant remaining after the precipitation of the hypotensive preparation was passed through the anion-exchanger EDE-10P in the formate form Each liter of the solution contained 700-800 mg of phosphorus, of which 600-700 mg was retained on the column measuring 50 cm × 1.5 cm^2. The material was eluted with 0.1 N formic acid and the effluent was collected in 250-300 ml fractions. Phosphorus was determined in each fraction according to Fiske and Subbarow. The results are presented in Table 1.

The terminal fractions were rejected and the remaining ones pooled and passed through a cation-exchanger washed free of formic acid until the pH of the eluate solution

TABLE 1. Phosphorus Content in Fractions Eluted From the Column of ÉDÉ-10P-Formate

Fraction No.	Fraction vol.	P content, mg
1	200	4.0
2	250	10.0
3	300	30.0
4	250	37.0
5	300	14.4
6	250	9.5
7	120	3.8

was 2.0-3.0. The cytidylic acid was retained, while small amounts of phosphorus representing some unidentified compounds passed through in the effluent. The cytidylic acid was eluted from the cation-exchange resin with 0.1 N formic acid. The eluate was colorless, all pigments having been retained on the column. The eluates were taken to dryness in vacuo at 40°. In order to remove the last traces of formic acid the residue was repeatedly taken up in water and dried. The residue was finally dissolved in the minimal amount of hot water and the solution poured into cold ethanol; a white, crystalline precipitate separated immediately. The suspension was allowed to stand in the cold, the material was filtered off and air-dried, yielding 680 mg; the material was dissolved in distilled water and filtered; the clear solution was evaporated to a small volume and allowed to crystallize in the cold. The crystallized product (fine long needles) was filtered off from the mother liquor, washed with ice-cold water, then with ethanol, and dried in vacuo over P_2O_5.

The purified preparation was checked by paper chromatography in two solvent systems: isobutyric acid–ammonia–water (66:1:33) and ethanol–ammonium acetate (pH 5.5) (70:30). In the two solvent systems used the compound moved as a single spot with R_f values 0.4 and 0.32, respectively. These values corresponded to those of authentic cytidylic acid. The absorption maximum in the ultraviolet was found to be at 273 mμ. The electrophoretic mobility of the compound in citrate buffer pH 3.7 corresponded with that of authentic cytidylic acid.

Found %: C 33.04; H 4.84; N 12.58; P 9.20
Calculated for $C_9H_{14}N_3O_8P$, %: C 33.45; H 4.37; N 13.00; P 9.60

The Isolation of Cytidylic Acid from an Alkaline Hydrolysate of Pancreatic Ribonucleoprotein:

The nucleoprotein (120 g) was hydrolyzed in 1.2 liters of 1 N NaOH for 24 hours at 37°. The insoluble residue was removed, washed with water and rejected. The washings were combined with the main filtrate. Moist cation-exchanger KU-2, in the H^+ form, was added to the mechanically stirred solution until the pH fell to 7.5-7.0. The resin was removed and washed with several portions of water. The washings were combined with the main filtrate. The solution was acidified to pH 5.0-4.5 with formic acid and allowed to stand in the cold. The bulky protein precipitate which separated out was removed and rejected. The clear, slightly colored filtrate still contained proteins as indicated by the Biuret reaction. A portion of the solution, containing 1100-1200 mg P in 2.5 liters, was passed through a column (50 cm × 5.5 cm^2) of the anion-exchanger ÉDÉ-10P-formate, washed free of formate until the effluent emerged at pH 2-2.7. The flow rate was 0.6 ml/min. The column, which retained the nucleotides, was not washed, but immediately eluted with 0.1 N formic acid. The eluate was collected in fractions which were assayed for phosphorus and also examined chromatographically in the solvent system: isobutyric acid–ammonia–water (66:1:33). The results are shown in Table 2.

The fractions containing cytidylic acid alone (172 mg P) were pooled and passed through a column (50 cm × 5.5 cm^2) containing the cation exchanger KU-2 (H^+ form), previously washed with 0.1 N formic acid. The column was eluted with 0.1 N formic acid. The first 760 ml of the effluent contained no phosphorus; the subsequent 250 ml

TABLE 2. Fractionation of Cytidylic Acid on a Column of ÉDÉ-10P-Formate

Fraction No.	Fraction vol.	P content, mg	Chromatographic analysis
1	300	12,5	Cytidylic acid
2	500	15,0	" "
3	300	77,5	" "
4	150	45,0	" "
5	120	22,0	" "
6	400	68,0	Cytidylic with adenylic contamin.
7	250	33,0	Cytidylic and adenylic acids
8	800	56,0	Adenylic and cytidylic acids
9	300	20,0	Adenylic acid with cytidylic contam.

contained only 3.25 mg P. These solutions were rejected. The subsequent fractions, containing 32, 64, 50, and 13.5 mg P respectively in each 400 ml, were pooled, evaporated to dryness under reduced pressure and the residue was freed from formic acid by repeated solution and evaporation. It was finally precipitated with acetone, which resulted in a viscous yellow mass. The latter was dissolved in 200 ml of 0.1 N formic acid and passed through a column (50 cm × 1.5 cm^2) of the cation-exchanger SDV-2 (H^+ form). The effluent contained no phosphorus and was rejected; the resin was then eluted with 0.1 N formic acid. One liter of 0.1 N formic acid was required to elute the entire cytidylic acid from the column. The combined eluates were taken to dryness, dissolved in a small volume of water and poured into cold alcohol. A flocculent white precipitate separated immediately. This suspension was allowed to stand in the cold overnight, the precipitate was centrifuged off and air-dried. Yield—1.45 g.

The crude material was recrystallized from water as described above, washed with alcohol and ether and dried to constant weight in vacuo over P_2O_5.

Found %: N 12.9; P 9.3.
Calculated for $C_9H_{14}N_3O_8P$, %: N 13.0; P 9.6.

The preparation was found to be chromatographically homogeneous and the location of the single spot corresponded to that of authentic cytidylic acid.

Fractions 6 and 7 of the effluents from the anion exchanger ÉDÉ-10P (Table 2), containing a mixture of cytidylic and adenylic acids, were rechromatographed on a column (50 cm × 1.54 cm^2) of ÉDÉ-10P-formate. This afforded a complete separation of the two nucleotides (see Table 3).

Passage of the pooled fractions through a column of the cation exchanger SDV-2 (H^+ form), followed by the usual purification steps, yielded an additional crop of 0.5 g of cytidylic acid.

TABLE 3. The Separation of Cytidylic Acid From a Mixture of Cytidylic and Adenylic Acids on a Column of EDE-10P-Formate

Fraction No.	Fraction volume	P content, mg	Chromatographic analysis
1	300	0	—
2	300	18.00	Cytidylic acid
3	100	10.00	The same
4	250	25.00	Cytidylic acid
5	100	8.5	The same
6	100	5.8	The same

DISCUSSION OF RESULTS

Nucleotide mixtures are conventionally fractionated on strongly basic anion-exchange resins. The domestic anion-exchange resins AN-2F and ÉDÉ-10, whose active groups are secondary and tertiary aliphatic amines (the anion exchanger ÉDÉ-10P, whose active groups include tertiary and quarternary ammonium groups, can be used as a reasonably strongly basin resin), differ from the strongly basic resins in that their retentive activity is restricted to a narrow pH range. Their retentive power is low at neutral pH, but increases with increasing acidity and in many cases exceeds the capacity of some strongly basic resins.

However, if the adsorption is carried out from acetone—water mixtures, the audity of the medium becomes less important in the determination of the degree of adsorption of the nucleotides on the above-mentioned anion-exchangers, although its affect is not entirely lost. Under those conditions the presence of salts or proteins in the solution is also of lesser importance than in pure aqueous solutions. When solutions in water-acetone mixtures were passed through columns containing ÉDÉ-10P or AN-2F all phosphorus-containing material was adsorbed on the resin and was thus separated from a large variety of other material. Under those conditions the capacity of the resin reached 20 mg P/g resin.

Solutions in aqueous acetone, used in the present work for the isolation of cytidylic acid, were usually strongly pigmented. The bulk of the colored material was adsorbed on the anion-exchanger on passing the solution through. The colored material was eluted, together with the nucleotides, with 0.1 N formic acid The highest concentration of the colored material coincided with the highest concentration of phosphorus in the fractions. This was used as an indirect indication of the progress of nucleotide elution. The eluate was thus a colored solution whose pH ranged from 2.0 to 3.0. This acid solution was passed through the cation-exchangers of the type SDV-2(H^+ form), or KU-2(H^+ form. The adsorption was most quantitative at pH 2.0.

Nucleotides, with the exception of uridylic acid, acquire a positive charge at pH values 2.0 and below [4], and are thus completely retained by cation-exchangers. In the case of incomplete adsorption the effluent was passed again through a fresh column. In the present experiments the capacity of the cation-exchangers was found to lie between four and six mg P/g resin.

Using the anion-exchanger ÉDÉ-10P (formate form), and eluting with 0.1 N formic acid, it was found possible to elute from the resin only the cytidylic acid, leaving other nucleotides, as well as inorganic P, on the column. The eluate from ÉDÉ-10P was passed through the cation-exchanger SDV-2 or KU-2. The desorption of nucleotides from cation-exchangers can be accomplished

with distilled water. However, the pigmented material emerged together with, or slightly ahead of, the nucleotide fraction. In order to prevent the simultaneous elution of the pigments with the nucleotides, elution was carried out with 0.1 N formic acid. The pigments were later removed from the cation-exchanger with aqueous alkali. It was also found that nucleotides eluted from SDV-2 considerably more readily than from KU-2.

The most convenient method for crystallizing cytidylic acid was found to be crystallization in the cold from a concentrated solution of the crude material in warm water. Within 48 hours the sides and bottom of the vessel became covered with a hard layer of the compound, consisting of uniform long needles. After the separation of the first, and also a second, crop from the mother liquor, a third crop of crystals was collected. These were of a different form, having the appearance of long, regular plates. Accoding to Loring and Lucy [5], the first type of crystals is the a isomer of cytidylic acid, and the second—the b isomer.

SUMMARY

The aqueous acetone supernatant obtained after the precipitation of the hypotensive hormone from saline extracts of the pancreas was shown to contain large amounts of cytidylic acid.

A simple procedure for the isolation and purification of cytidylic acid from ribonuclease and alkaline hydrolysates of pancreatic RNA has been developed. The procedure involves the use of domestic ion-exchange resins—the anion-exchanger EDE-10P (formate form) and the cation-exchangers SDV-2 or KU-2 (H^+ form. The use of the cation-exchangers with an elution step involving 0.1 N formic acid afforded a separation of the nucleotide from pigment material without the use of charcoal or multiple recrystallization.

LITERATURE CITED

[1] R.J. Harris and J.F. Thomas, J. Chem. Soc. 1936, (1948); Nature 161, 931 (1948).

[2] S.V. Andreev, N.P. Speranskaya, I.D. Kobkova, and N.I. Gavrilov, Doklady Akad. Nauk SSSR 105, 848 (1955).

[3] B. Magasanik, E. Vischer, and R. Doniger, et al., J. Biol. Chem. 186, 37 (1950).

[4] W. Cohn, J. Amer. Chem. Soc. 72, 1471 (1950).

THE RATE OF CHOLESTEROL AND FATTY ACID SYNTHESIS IN THE ADRENALS, TESTES AND LIVER OF YOUNG AND OLD RATS, BOTH NORMAL AND IRRADIATED

K.A. Tret'yakova and D.É. Grodzenskii

Radiation Laboratory, All-Union Institute of Experimental Endocrinology, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp 399-403,
May-June, 1960
Original article submitted July 30, 1959

Numerous researches have shown that with aging there is a change in the morphological structure of endocrine glands, which leads to a lowering of functional activity [1] and disturbance of lipid metabolism [2-6].

Starting from this, it was decided to compare the rate of cholesterol and fatty acid synthesis in endocrine glands and the liver of young and old rats of both sexes and to check whether increase in cholesterol synthesis was characteristic of old animals, for this was noticed earlier in young animals after action of ionizing radiation. In available literature there could not be found any data concerning the influence of age on the rate of cholesterol and fatty acid synthesis, even though it has been established that irradiation considerably increased cholesterol and fatty acid synthesis in adult rats [7-12]. As is known, cholesterol is a precursor of steroid hormones and in relation to this the investigation of cholesterol synthesis in adrenals and testes assumes special importance.

METHODS

Experiments were conducted on rats of both sexes and of three age groups. Group I - young animals, one and a half months of age and weighing 100 g, Group II - -animals of about 20 months of age, weighting 250-350 g, Group III - males, 30 months of age, weighing 350- -450 g and females, 23 months of age, weighing 300- -350 g.

TABLE 1. Relative Organ Weights of Rats of Different Ages. Organ Weights Per 100g of Body Weight

Experimental animals				Organ weight		
group	weight, g	age, months	number	liver, g	adrenals, mg	testes, g
Intact males						
I	100	1½	20	3,73	25,2	1,38
II	250—350	20	8	2,72	15,00	0,89
III	350—450	30	9	2,64	13,80	0,73
Irradiated males						
III	350—450	30	10	2,86	20,3	0,71
Intact females						
I	300	1½	12	3,80	33,80	—
II	250—350	20	8	3,06	30,10	—
III	300—350	23	6	2,78	26,20	—
Irradiated females						
III	300—350	23	6	3,03	24,70	—

Isotope methods were used for investigation of the rate of cholesterol and fatty acid synthesis. The animals were starved for 20 hrs, and then injected subcutaneously with $CH_3C^{14}OOH$ at the rate of 25,000-35,000 cpm/g weight. After four hours they were bled to death. Isolation of labeled cholesterol and fatty acid was conducted according to methods previously described by the authors [7, 8] and also by Bolker [13]. Rats of group III were irradiated under standard conditions (17 ma, 180 kv, filter 1 mm Al and 0.5 mm Cu, dose strength 40 r /min) with a total dose of 800r. Immediately after irradiation the rats were starved for 20 hrs, and then were injected subcutaneously with $CH_3C^{14}OOH$ at the rate of 25,000 cpm/g. The rats were killed after four hours. In this way cholesterol synthesis was investigated 24 hours after irradiation.

Radioactivity of cholesterol and fatty acids was studied from thin layers in a block counter. Total cholesterol was determined by the method of Sperry and Webb [14], and total number of fatty acid by weighing.

TABLE 2. Cholesterol and Fatty Acid Content in Organs of Young And Old Rats, Normal and 24 Hours After X-ray Irradiation

Group	Sex	Weight, g	Age, months	Number of animals	Liver		Adrenals	Testes	
					total cholesterol (in mg % ± m)	total fatty acids (in % ± m)	total cholesterol (in % ± m)	total cholesterol (in mg % ± m)	total fatty acids (in % ± m)
Intact animals									
I	Males	100	1½	18	279±7,0	2,63±0,1	1,700±0,16	219±6,85	1,95±0,14
II	"	250—350	20	8	318±8,0	3,23±0,1	2,427±0,11	194±3,3	1,57±0,17
I	Females	100	1½	11	316±9,85	3,84±0,35	2,171±0,34	—	—
II	"	250—350	20	14	316±6,05	3,97±0,33	2,720±0,26	—	—
Irradiated animals									
Control	Males	350—450	30	9	331±15,2	4,75±0,27	2,62 ±0,53	219±0,68	1,61±0,11
Experiment	"	350—450	30	10	303±11,9	5,02±0,27	1,707±0,34	215±4,7	1,38±0,04
Control	Females	300—350	23	14	316±6,05	3,97±0,33	2,72 ±0,26	—	—
Experiment	"	300—350	23	6	298± 8,10	5,35±0,47	1,94 ±0,39	—	—

Note. Standard error $m = \pm \sqrt{\frac{\Sigma a^2}{n(n-1)}}$.

RESULTS

Attention is directed to differences in organ weights of rats in various age groups (Table 1).

It became evident that with age the relative organ weights decreased. After irradiation the adrenal weights of old males increased sharply. Liver weights were increased in rats of both sexes, but to a lesser degree. Adrenal weights in females were somewhat decreased.

In Table 2 are presented data concering investigations into the cholesterol content of different organs in the various age groups.

Data in Table 2 show that, in comparison with young rats, the liver cholesterol content of old males was somewhat higher, and in testes lower; in females it was unchanged. In adrenals of old rats of both sexes the cholesterol content was considerably higher than in young ones. These data were statistically significant. Twenty-four hours after irradiation of old rats with the dose of 800 r there was a statistically significant decrease in cholesterol content of the adrenals. In Table 3 are presented data concerning the specific activities of cholesterol and amino acids isolated from organs of rats in groups I and II. There was no pronounced difference in the rate of cholesterol and fatty acid synthesis in rats of various ages. In the testes of old rats only was the cholesterol specific activity significantly lower than in testes of young ones. The difference in liver cholesterol specific activity of males and females was related to the unequal amount of radioactivity introduced per gram of weight into the different sexes.

In Fig. 1 there is shown the influence of x-rays on the rate of cholesterol synthesis in old rats. After irradiation, the rate of cholesterol synthesis in the liver was increased by 550-830%; in the adrenals, - by 445--390%. In the testes the rate of cholesterol synthesis was changed insignificantly. Changes in the rate of fatty acid synthesis after irradiation are shown in Fig. 2. If in the normal liver the specific activity of fatty acids was equal to 10 ± 0.73 cpm/mg then after irradiation, data were obtained for liver from females. In the testes the specific activity of fatty acids after irradiation remained unchanged.

DISCUSSION OF RESULTS

In the organism of old rats (20 months) in comparison with young rats (1.5 months) there were a number of changes. These changes concerned organ weights, rate of cholesterol synthesis in the liver and testes, and also cholesterol content. Lowering of endocrine weight in old animals [15] and also the change in cholesterol content was also observed by other authors [2, 3]. But investigations devoted to the study of the influence of age on the rate of cholesterol and fatty acids formation are unknown to the authors. According to the literature, the main place of cholesterol synthesis in the organism is the liver. According to the present data the weight of the liver decreases with age, its cholesterol content increases, and biosynthesis of cholesterol shows a tendency to increase. These facts attest that in the liver of old

TABLE 3. Specific Activity of Cholesterol and Fatty Acids (in cpm/mg ± m) From Organs of Rats of Various Ages. Rats starved for 20 hrs. They were injected subcutaneously with $CH_3C^{14}OOH$ and killed four hours later. Figures in parenthesis - - number of experiments.

Animal group	Weight, g	Liver		Testes	Adrenals	
		males*	females**		males*	females**
Cholesterol						
I	100	191±33,8 (12)	99±18,0 (12)	23±2,62 (12)	46±12 (6)	47±6,4 (8)
II	250—350	279±65,5 (8)	111±25,1 (8)	13±2,05 (8)	42±10,7 (8)	33±7,0 (9)
Fatty acids						
I	100	16±1,16 (11)	10±1,40 (11)	30±2,66 (12)	—	—
II	250—350	18±3,69 (8)	9±0,90 (8)	33±2,81 (8)	—	—

* Injected with 35,000 cpm/g.
** Injected with 25,000 cpm/g.

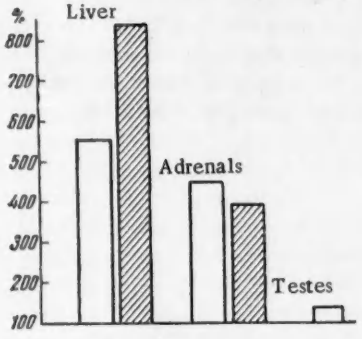


Fig. 1. Increase in the rate of cholesterol synthesis in organs of old rats after irradiation (800r) in % of original. Unshaded columns - males; crosshatched columns - females.

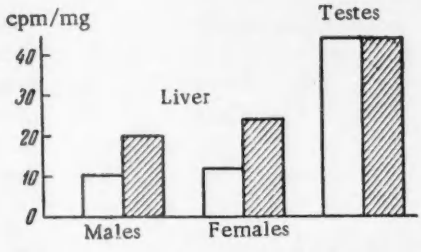


Fig. 2. Rate of fatty acids synthesis in organs of normal and irradiated old rats. Unshaded columns - controls; crosshatched columns - experimental.

rats there is a fully effective enzyme system responsible for cholesterol synthesis. One of the reasons for the increase in testicular cholesterol levels of old rats, with a concurrent stability in the rate of cholesterol synthesis, could be a decrease in utilization of cholesterol in this organ; for example, lowering of the formation of steroid hormones from cholesterol.

Reduction of testes weight, lowering of their cholesterol content and lowering of the degree of C^{14} incorporation into cholesterol in old rats as compared to young ones, signals the extinction of the function of this organ. Larsson in his work [16] observed the reduction of testicular function in 22 months old rats.

Following total irradiation of old rats by a dose of 800r there was noticed an increase in adrenal weights of the males and a reduction of the cholesterol content, and likewise, an increase in intensity of cholesterol synthesis in the liver and the adrenals (5-8 fold). Cholesterol synthesis in the indicated organs is closely related to hypophyseal function [17-19]. Analyses of the experimental results point to the fact that all the animals respond to x-ray irradiation in the same way as young animals [7-8], i.e., basically there is an intensification of cholesterol and fatty acid synthesis.

In accordance with literature data, the most important factor in accelerating cholesterol synthesis following irradiation is the acceleration of the initial stages of cholesterol synthesis from acetic acid [10-12]. However, the accelerated mechanism of cholesterol synthesis following irradiation and the meaning of this fact in pathogenesis of radiation injury remains unclear. Further investigations by the present authors are directed toward clarification of the reasons for the increase in cholesterol synthesis under the influence of ionizing radiation.

SUMMARY

The relative weights of adrenals, testes and the liver of old rats was lower in comparison to young rats. The cholesterol content of the liver and the adrenal of old rats was increased in the testes it was decreased. The rate of cholesterol synthesis in organs of old rats was basically not different from the rate in young animals, with the exception of the testes. In the testes the rate was lowered. A day after x-ray irradiation with a minimal absolute lethal dose, the cholesterol content of ardenals was lowered in old rats of both sexes. The

specific activity of cholesterol isolated from the liver and the adrenals of old rats of both sexes, a day after irradiation, was considerably higher than in control animals. The specific activity of cholesterol and fatty acid isolated from the testes of old rats following irradiation was unchanged. X-ray irradiation accelerated fatty acid synthesis in the liver of old rats of both sexes.

LITERATURE CITED

[1] E.A. Vasukova, Med. Rabotnik 72 (1959).

[2] V.I. Solntzev in: Old Age [in Russian] (Kiev, 1939), p. 395.

[3] M.D. Gantzanuk in: Old Age [in Russian] (Kiev, 1939), p. 221.

[4] L. Brull and C. Keil in: Experimental Research on Aging (1956), p. 83.

[5] G.R. Walker, E.H. Morse, and M. Potgieter, J. Nutrition 60, 517 (1956).

[6] H.S. Weiss, Proc. Soc. Exptl. Biol. and. Med. 95, 487 (1957).

[7] D.E. Grodzenskii, Med. Radiologiya 5, 77 (1959).

[8] K.A. Tret'yakova and D.E. Grodzenskii, Vopr. Med. Khimii, 5, 362 (1959).

[9] R.G. Gold, V.L. Lolz, and E. Lilly, Federat. Proc. 15, 264 (1956).

[10] R.G. Gold and G. Popjak, Biochem. J. 66, 51 (1957).

[11] N. Bucher, A. Loud and K. McGarrahan, Federat. Proc. 16, 17 (1957).

[12] N. Bucher, K. McGarrahan, E. Gould, and A. Loud J. Biol. Chem. 234, 262 (1959).

[13] H.J. Bolker, S. Fischman, and R.D. Heard. et al., J. Exptl. Med. 103, 199 (1956).

[14] W. M. Sperry and M. Webb, J. Biol. Chem. 187, 97 (1950).

[15] N.A. Shereshevskii in: Old Age [in Russian] (Kiev, 1939), p. 31.

[16] K. Larsson, J. Gerontology 13, 70, (1958).

[17] R. Rosenman, M. Friedman, and S.O. Byers. Endocrinol. 51, 142 (1952).

[18] G.V. LeRoy, R.G. Gould, and D.M. Bergenstal, et al., J. Labor. and Clin. Med. 49, 858 (1957).

[19] G.M. Tomkins, J.L. Chaikoff and L.L. Bennett, J. Biol. Chem. 199, 543 (1952).

THE TRANSFORMATION OF FLAVONOIDS IN TEA PRODUCTION

M.A. Bokuchava and N.I. Skobeleva

A.N. Bakh Biochemical Institute, Academy of Sciences, USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 404-406,
May-June, 1960
Original article submitted August 4, 1959

Our preceding publication [1] was devoted to the problem of increased vitamin P value and improved quality of tea. In it a study was made relating to the possibility of increasing the vitamin P content of tea by raising its catechin content. The possibility was demonstrated of obtaining a superior-quality tea with a higher catechin, and thus also vitamin P-content by means of regulating the biochemical processes during its processing.

Since the vitamin P properties of tea depend largely not only on catechins but also on flavonoids, it was considered worth while to undertake a study of these substances in the process of tea manufacture. Our objective consisted in a study of the flavonoid content in the available raw material, as well as in tea obtained by the established and the new technological procedures. The new technology consists basically in allowing a shorter period for the action of the oxidative ferments, and in applying a thermal treatment to the underfermented semifinished product.

The determination of flavonoids was conducted by Wilson's method, as modified by Guseva and Nestyuk [2]. The results of flavonoid determinations in tea leaf, as well as in the finished product obtained therefrom, are given in Table 1.

According to the data of Table 1, the amount of flavonoids is considerably reduced in the process of tea manufacture. According to the established technological process it amounts to 40%, and for the new process, 20-25%.

TABLE 1 The Content of Flavonoids in the Green Leaf and Semifinished Product,* Processed According to the Established (Control) and the New (Experimental), Method During the 1958 Season

No	Fraction	Flavonoids in % of dry weight	No	Fraction	Flavonoids in % of dry weight
864	Issuing green fixed leaf	1.67	1497	Issusing green fixed leaf	1.60
864	I Control	0.80	1497	II Control	0.60
864	I Experimental	0.93	1498	II Experimental	0.63
566	Issusing green fixed leaf	1.3	1100	Issusing green fixed leaf	1.60
566	II Control	0.74	1100	III Control	0.80
566	II Experimental	1.07	1100	III Experimental	1.20

*Obtained from the Dagomyss tea factory.

This is explained by the fact that in the established process there takes place a comparatively extended fermentation of the tea leaf, while in the new process the duration of the fermentation period, from the beginning of curling to the end of drying, is shortened from two to three times. Here the second fermentation phase is entirely left out, being replaced by the thermal treatment of the semifinished product. This permits the preservation of a higher catechin content [1], as well as of flavonoids (Table 1).

The Flavonoid Content of Teas of Various Origin. In view of the introduction of a new technology in various tea-producing regions of the USSR, it seemed of interest to conduct a study of the flavonoid content of tea brands produced by the new and the established methods in the factories of Georgia, Azerbaidzhan and Krasnodar districts.

The data of Table 2 show that in commercial tea brands there are more flavonoids in the tea processed by the new technique than by the current one.

The Flavonoid Content as Related to The Age of The Tea Leaf. It is known that the amount of polyphenolic compounds varies greatly depending on the age of the leaf. It would, thus, be of interest to conduct a study of the flavonoids in tea leaves of various ages.

The investigation was conducted on fixed two-leaf twigs of ten-twelve days of age, fixed mature leaves of

TABLE 2. The Content of Flavonoids in Commerial Brand Tea, Processed According to the Established (Control and the New (Experimental Method During the 1958 Season

Anaceul tea factory Georgian SSR				Astarin tea factory Azerbaidzhan SSR			
trade mark	kind of leaf	method	flavonoids in % of dry weight	trade mark	kind of leaf	method	flavonoids in % of dry weight
13	L - 1 *	Established	0.54	4	L - 1	Established	0.61
1	L - 1	New	0.86	7a	L - 1	New	0.93
19	M - 1 *	Established	0.85	1	MS - 1	Established	0.94
17	M - 1	New	1.07	4a	MS - 1	New	1.00
17	L - 2	Established	0.66	9	MS - 2	Established	0.86
51	L - 2	New	0.80	3a	MS - 2	New	0.93

*L - leaf tea; MS - small particle tea

TABLE 3. The Content of Flavonoids in the Green Leaf and Commercial Brand Tea of Various Regions of India

Kind under investigation	Region	Flavonoids in % of dry weight
Green fixed leaf (two-leaf twig)	Assam	1.17
VOR Brand of tea	"	0.95
Green fixed leaf (two-leaf twig)	Kunur	1.67
VOR Brand of tea	"	1.20
Green fixed leaf (two-leaf twig)	Darjeeling	1.13
VOR Brand of tea	"	1.03

forty to forty-five days of age, and fixed old leaves. They contained the following amounts of flavonoids:

Material	Flavonoids in % of dry weight
Two-leaf twigs	1.13
Mature leaves	1.74
Old leaves	1.01

Therefore, mature leaves contain the largest amounts of flavonoids.

The Flavonoid Content in Green Tea Leaf and in Commercial Brand Tea From Various Regions of India. The flavonoids were investigated also in green tea leaf and in finished tea from various regions of India. One can see from Table 3 that a regular decrease of flavonoids during the processing of tea leaf takes place also under the conditions of tea manufacture in India.

It is, however, significant that the percent of decrease in the flavonoid substances during processing of the tea leaf in India is much less than under our technological procedure. In this respect, our tea brands, when prepared according to the new procedure, come close to the India brands in flavonoid content.

SUMMARY

A study of the flavonoid content in the green tea leaves and in the finished tea, produced by the established as well as the new procedure, shows that there is a marked decrease in flavonoids during the process of its manufacture, and that in tea produced by the new procedure this decrease is of lesser magnitude. This is explained by the fact that while the established technology involves a comparatively prolonged fermentation of the tea leaf, the new technology implies a from two to three times shorter fermentation process, from the beginning of curling to the drying endpoint.

The high flavonoid content in the tea prepared by the new process increases its vitamin P and physiological value.

The processing of tea leaf in India lowers the flavonoid content much less as compared with our established procedure. When prepared by the new technology, our brands of tea approach in flavonoid content those of India.

LITERATURE CITED

[1] M.A. Bokuchava, N.I. Skobeleva, and A.M. Knyazeva, Biokhimiya 24, 371 (1959).*

[2] A.R. Guseva and M.N. Nestyuk, Biokhimiya 18, 480 (1953).

*Original Russian pagination. See C.B. Translation.

THE REGULATORY SIGNIFICANCE OF THE DPN$^+$–DPNH SYSTEM IN INTERMEDIATE CARBOHYDRATE METABOLISM*

A.I. Kolotilova

Department of Biochemistry, A.A. Zhdanov State University, Leningrad

Translated from Biokhimiya Vol. 25, No. 3, pp. 407-416,
May-June, 1960
Original article submitted August 11, 1959

It is well known that the transition of an animal organism from a state of comparative rest to one of activity is associated with a change in metabolic rates of its cells and tissues. The mechanism by which these rates are controlled is, however, still very obscure.

The rate of an enzyme reaction depends, within definite limits, on the concentration of the interacting components – the substrate, coenzyme (if the reaction is catalyzed by a conjugated protein), and the enzyme. In reversible reactions, with a given enzyme concentration, a change in concentration of substrate or coenzyme may result not only in a change in rate or, according to the terminology of Englegardt [1], a change in molar activity, but also in a reversal of the reaction. This concentration effect is apparently one of the mechanisms of regulation of metabolic rate at the cell level.

Among the easily-reversible reactions of the anaerobic stage of carbohydrate metabolism are those catalyzed by dehydrogenases with concomitant changes in the diphosphopyridine nucleotides.

In previous studies [2-4] with erythrocytes at physiological levels of enzymes and coenzymes, there was found, in addition to hexokinase and phosphofructokinase activity, a small activity due to triose phosphate dehydrogenase which was associated with synthesis of ATP and formation of DPNH.

In model experiments Neifakh and Mel'nikova [5], using high concentrations of substrate, coenzyme, enzyme, and stabilizers and activators, convincingly showed that the rate of glycolysis is increased by addition to a dilute dialyzed muscle extract of hexokinase and phosphofructokinase, which have a small activity in intact muscle. These experiments confirmed the results of earlier studies of the limiting role of hexokinase and phosphofructokinase in glycolysis [6-8]. Addition of triose phosphate dehydrogenase under the same conditions did not increase glycolytic rate.

Here it might be concluded that this enzyme is not able to exert a regulatory influence on glycolysis. However, these experiments were performed at high levels of substrate and coenzyme. Ths possibility of regulating glycolytic rates by altering concentrations of the reactants under more physiological conditions therefore remains open.

In this study, the rates of those reactions catalyzed by triose phosphate dehydrogenase, α-glycerophosphate dehydrogenase, and lactic dehydrogenase were followed spectrophotometrically. Starting substrates were obtained by the aldolase reaction.

The purpose of the experiments was to determine the effect of concentration of substrate and coenzyme (DPN$^+$ and DPNH) within physiological limits on rates of reactions catalyzed by these enzymes [9].

MATERIALS AND METHODS

Dibarium salt of fructose-1,6-diphosphate was synthesized according to the usual procedure [10] by yeast fermentation of glucose using bottom-fermenting yeast. The compound was used in the form of the sodium salt. Analysis of fructose in the preparation indicated it to be 70% pure.

Phosphotrioses. A mixture of 3-phosphoglyceraldehyde and dihydroxyacetone phosphate was obtained by the action of aldolase on fructose-1,6-diphosphate. Phosphotriose content was determined in a protein-free extract (protein coagulation by trichloracetic acid or by heating 1 minute at 100°) by the Fiske-Subbarow method according to the increase in alkali-labile phosphorus. Optical density was determined on an FÉK-M colorimeter.

Lactic and pyruvic acids were commercial preparations and were used in the form of their sodium salts. The DPNH preparation used, of 56% purity, was also a commercial preparation.

DPN$^+$ was obtained by the interaction of DPNH and dihydroxyacetone phosphate. In addition, a preparation of 50% purity was used.†

Triose phosphate dehydrogenase, or 3-phosphoglyceraldehyde dehydrogenase, was isolated in crystalline form from rabbit skeletal muscle by the method of Cori, Slein, and Cori [11] as modified by Dandliker and Fox

* The following abbreviations for compounds were used: ATP -- adenosine triphosphate; DPN$^+$ -- oxidized form of diphosphopyridine nucleotide; DPNH -- reduced form of diphosphopyridine nu nucleotide; EDTA -- ethylenediamine tetraacetate.

† We are indebted to Professor I.A. Rappaport (Berlin) for the gift of the DPN$^+$ preparation.

[12], who suggested that to stabilize the enzyme all reagents used in its isolation be prepared in a 0.002M solution of the disodium salt of EDTA.

Aldolase was obtained in amorphous form from rabbit skeletal muscle by the method of Taylor, Green, and Cori [13]. The preparation also contained α-glycerophosphate dehydrogenase, which catalyzes the oxidation of DPNH to DPN+ and the reduction of dihydroxyacetone phosphate to α-glycerophosphate. The possibility of a similar association of enzymes has been noted by a number of workers [14-17].

Lactic dehydrogenase was isolated in purified form from ox heart muscle by the method of Neilands [18]. Aldolase and triose phosphate dehydrogenase preparations were stored under $(NH_4)_2SO_4$ mother-liquor solutions of appropriate concentrations at 0-4°. Lactic dehydrogenase was also stored as a frozen solution in the refrigerator after dialysis against phosphate buffer, pH 7.0. Enzyme preparations‡ were kept for months without perceptible change in activity.

Enzyme concentration was determined in an SF-4 spectrophotometer by absorption at 280 mμ. Concentrations were calculated from the formula $C=E_{280}$ K1, where C is enzyme concentration (in mg/ ml) of the sample, E_{280} the extinction of the enzyme solution at 280 mμ, K the extinction coefficient at 1 mg/ml of enzyme, and the thickness of the cuvette (in cm). K of aldolase was taken as 0.8 [13], of lactic dehydrogenase as 1.5 [18], and of triose phosphate dehydrogenase as 1.0 [19].

Reactions catalyzed by triose phosphate dehydrogenase, α-glycerophosphate dehydrogenase, and lactic dehydrogenase were followed by observing changes in DPNH content. DPNH was determined at 340 mμ in rectangular cuvettes 1 cm thick. For calculation of DPNH concentration, its molar extinction was taken as $6.22 \cdot 10^{-6}$ cm^2 · · moles^{-1} in accordance with the data of Horecker and Kornberg [20].

The conversion of the extinction value to concentration of DPNH was made according to the following equation:

$$X = \frac{E_{340} \cdot V}{0.00937 \cdot 663},$$

where X is the amount of DPNH in the sample expressed in μmoles, E_{340} the extinction of the sample at 340 mμ, V the volume of the sample in ml, 0.00937 the extinction of a solution containing 1 μg DPNH per ml [20], 663 the molecular weight of DPNH.

EXPERIMENTAL PROCEDURE

The aldolase reaction was carried out at concentrations of fructose-1,6-diphosphate from 1 to 10 μmoles per ml of sample. Aldolase concentrations ranged from 2 to 8 mg per ml of sample. Sample volume varied from 1 to 3 ml. Incubation time varied from 15 to 60 minutes.

α-Glycerophosphate dehydrogenase activity was followed by measuring the decrease in DPNH added to the products of the aldolase reaction. Amounts of DPNH used in separate experiments varied from 0.07 to 0.3 μmoles per ml of sample.

The triose phosphate dehydrogenase reaction was followed in the reaction mixture consisting of products of the aldolase and the α-glycerophosphate dehydrogenase reactions. In accordance with published procedures [11, 15, 21, 22], this reaction was studied after addition to the samples (in final concentration) of $2 \cdot 10^{-4}$ M sodium pyrophosphate, $1.3 \cdot 10^{-4}$ M sodium arsenate, and $2.6 \cdot 10^{-4}$ M cysteine or glutathione.

The reaction was begun by introduction of the enzyme in concentrations of 0.3-0.7 mg per ml of sample.

In the presecne of arsenate, the reaction product is not 1,3-diphosphoglyceric acid but 3-phosphoglyceric acid. Under these conditions, 1-arseno-3-phosphoglyceric acid formed by the oxidation of 3-phosphoglyceraldehyde is rapidly decomposed to yield arsenate and 3-phosphoglyceric acid. The decomposition of the original reaction product renders the reaction irreversible, since ATP and the corresponding phosphokinase are absent from the reaction mixture [23].

Oxidation of lactic acid to pyruvic acid was carried out in glycine buffer, pH 10, in a system consisting of lactic dehydrogenase, DPN^+, and lactate [18]. Lactate content of the samples varied from 0.5 to 50 μmoles, DPN^+ content from 100 to 200 μg, and lactic dehydrogenase content was 0.02 mg. Sample volume was 3.22 ml, and experiments were continued for 40 to 130 minutes. Experiments with α-glycerophosphate dehydrogenase, triose phosphate dehydrogenase, and lactic dehydrogenase were carried out at room temperature, about 20°, and those with aldolase at 30 to 37°.

RESULTS

Aldolase Reaction

In experiments with various concentrations of fructose-1,6-phosphate, a linear relation between substrate concentration and increase in phosphotriose was obtained. Within the substrate concentration range studied (1-10 μmoles/ ml), 0.5 to 2.0 μmoles of phosphotriose were formed.

TABLE 1. Content of Inorganic and Alkali-Labile Phosphorus in Aldolase Reaction Mixtures Under Various Experimental Conditions

Exptl. conditions	Phosphorus in μmoles per ml of sample	
	inorganic	alkali-labile
Before incubation	0.26	0.11
After 60 minutes' incubation at 30°	0.36	1.95
After 60 minutes' incubation at 30° and 1 minute at 100°	2.61	2.00

‡We are indebted to O.D. Migulevaya for her help in the preparations.

To free the reaction mixture of protein, samples were heated 1 minute at 100°, then cooled and centrifuged to remove the coagulated aldolase preparation. The effect of such a treatment on sample composition is shown in Table 1.

Table 1 shows that heating at 100° caused a marked increase in inorganic phosphorus and a moderate increase in alkali-labile phosphorus, this fraction being as high as 2.0-3.0 μ moles per ml with such treatment. The increase in the inorganic phosphorus fraction after heating at 100° is evidently related to the activity of phosphatases contaminating the aldolase preparation. The initial increase in temperature during the heating period apparently induced an increase in rate of decomposition of phosphoric esters by these enzymes. Addition of DPNH had no substantial effect on either fraction.

α-Glycerophosphate Dehydrogenase Reaction

As indicated earlier, the aldolase preparation also contained α-glycerophosphate dehydrogenase activity. This activity was manifested by a reduction in amount of DPNH added to the reaction mixture containing products of aldolase action.

Addition of DPNH to a sample containing only fructose-1,6-diphosphate or only an aldolase preparation resulted in an increase of the extinction at 340 mμ which was proportional to amount of DPNH added. When an aldolase preparation was added to a sample containing fructose-1,6-diphosphate and DPNH, however (Table 2), there was a sharp decrease in the extinction value (expt. I, sample 1), and when DPNH was added to a solution of phosphotrioses and aldolase there was only a slight increase in the extinction value (expt. II, sample 1). Apparently dihydroxyacetone phosphate formed by the action of aldolase on fructose-1,6-diphosphate has rapidly oxidized DPNH to DPN^+, being itself reduced to α-glycerophosphate. This reaction came to equilibrium during the first few seconds, was sensitive to heat, and ceased after 1 minute in boiling water.

Triose Phosphate Dehydrogenase Reaction

The triose phosphate dehydrogenase reaction was carried out in samples after the aldolase and α-glycerophosphate dehydrogenase, and DPNH. Products being formed by the action of these enzymes were 3-phosphoglyceraldehyde, dihydroxyacetone phosphate, α-glycerophosphate, DPN^+ with a very slight admixture of DPNH; residual unreacted fructose-1,6-diphosphate was also present.

In most of the experiments with triose phosphate dehydrogenase these samples were used after heating at 100° and removal of the coagulated aldolase preparation. Some experiments were performed without preliminary removal of the enzymes.

Results of a typical experiment carried out after removal of aldolase are presented in Fig. 1. In the process of 3-phosphoglyceraldehyde oxidation an equivalent amount of DPNH is formed and this is reflected in the increase in extinction at 340 mμ. Reaction rate is low at a low starting concentration of fructose-1,6-diphosphate (~1 μ mole/ml) and rises sharply with an increase in fructose-1,6-diphosphate (to 4 μ moles/ml). Further increase in the original substrate was not accompanied by a corresponding increase in rate of the

TABLE 2. The Oxidation of DPNH in Samples Containing Fructose-1,6-Diphosphate, DPNH, and an Aldolase Preparation Contaminated with α-Glycerophosphate Dehydrogenase. Sample Composition: Expt. I. 2.3 μ moles Fructose-1,6-diphosphate; 0.2 μ moles DPNH, and 2.7 mg enzyme, temp. 20°. Expt. II. 1.7 μ moles fructose-1,6-diphosphate; 1.1 μ mole DPNH, and 4.6 mg enzyme, temp. 20°

Expt. no	Sample no.	Sample composition	Volume, ml	ΔE_{340}
I	1	Fructose-1,6-diphosphate + DPNH	2.6	–
		The same + enzyme, immediately	2.7	−0.430
		The same + enzyme, after 20 minutes	2.7	−0.435
	2	Fructose-1,6-diphosphate + DPNH	2.6	–
		The same + water, immediately	2.7	0.010
		The same + water, after 20 minutes	2.7	−0.005
	3	Water + DPNH	2.6	–
		The same + enzyme, immediately	2.7	−0.053
		The same + enzyme, after 20 minutes	2.7	−0.083
II	1	Fructose-1,6-diphosphate + enzyme	3.4	–
		The same (3 ml) + DPNH (0.5 ml), after 5 minutes	3.5	+0.250
		The same, after 20 minutes	3.5	+0.200
	2	Water + enzyme	3.4	–
		The same (3 ml) + DPNH (0.5 ml) after 5 minutes	3.5	+1.805

triose phosphate dehydrogenase reaction. Evidently fructose-1,6-diphosphate concentration was no longer limiting the rate of this reaction. In this event, DPN^+ became the limiting factor.

In those experiments performed without preliminary heating to inactivate aldolase and α-glycerophosphate dehydrogenase, an extremely small increase in extinction at 340 mμ was observed. Triose phosphate dehydrogenase reaction rates in the presence and the absence of an aldolase preparation are illustrated in Fig. 2. When the aldolase preparation is present, the effect of the admixture of α-glycerophosphate dehydrogenase is manifested in

Fig. 1

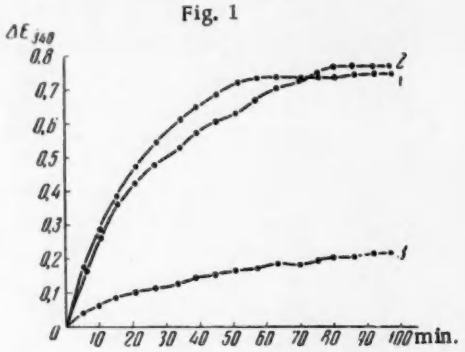


Fig. 2

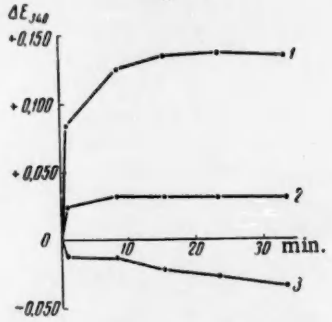


Fig. 1. The effect of fructose-1,6-diphosphate concentration on the triose phosphate dehydrogenase reaction. Starting amounts of fructose-1,6-diphosphate in μ moles: 1) 8.6; 2) 4.3; 3) 0.9; DPNH, oxidized in the α-glycerophosphate dehydrogenase reaction to DPN^+, 1.35 μ moles in all samples. Sample volume 2.5 ml.

Fig. 2. The effect of the aldolase preparation on the triose phosphate dehydrogenase reaction. 1) Fructose-1,6-diphosphate + aldolase + DPNH; triose phosphate dehydrogenase added after incubation at 30°, heating at 100°, and centrifugation; 2) the same, without heating at 100°; 3) aldolase + DPNH + triose phosphate dehydrogenase without heating at 100°. Starting amounts in samples: fructose-1,6-diphosphate – 4.1 μ moles; DPNH – 0.36 μ moles; aldolase – 5.8 mg; triose phosphate dehydrogenase – 1.8 mg. Sample volume 3.0 ml.

the oxidation of DPNH arising from the oxidation of 3-phosphoglyceraldehyde.

The experiment carried out with triose phosphate dehydrogenase in the absence of sodium arsenate (Fig. 3) at physiologically low starting substrate levels shows that, under these conditions, when the reaction is reversible, almost no perceptible increase in DPNH can be detected.

Lactic Dehydrogenase Reaction

Control experiments showed that in the absence of substrate or enzyme there was a slight increase in the extinction value. Only in a complete system, however, (lactate, DPN^+, and lactic dehydrogenase), was there a significant regular increase indicating the accumulation of DPNH. The dependence of reaction rate on concentration of lactate and of DPN^+ is shown in Figs. 4 and 5. At low concentrations of these reactants, this dependence is linear.

Since the H^+ ion is formed together with DPNH in this reaction, pH has an effect on reaction course. As Fig. 6 shows, acidification of the reaction medium reversed the reaction from lactate oxidation to pyruvate

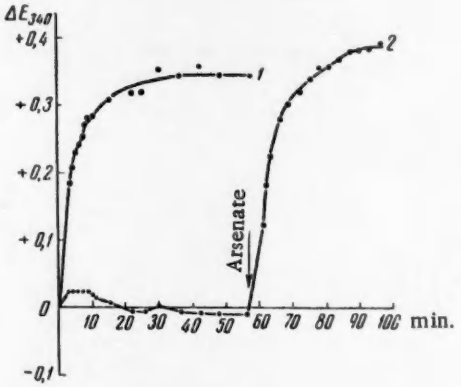


Fig. 3. The effect of arsenate on the triose phosphate dehydrogenase reaction. 1) With sodium arsenate; 2) arsenate added after 57 minutes. Starting amounts in samples: fructose-1,6-diphosphate – 4.1 μ moles; DPNH – 0.36 μ moles; arsenate – – 0.2 ml, $2 \cdot 10^{-3}$ M. Sample volume 3.1 ml.

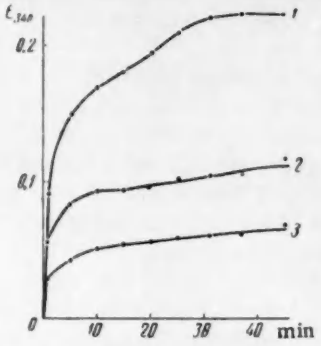


Fig. 4. The effect of lactate concentration on the lactic dehydrogenase reaction. Sample composition: lactate + + DPN^+ + lactic dehydrogenase. Lactate content of samples in μ moles: 1) 50.0; 2) 5.0; 3) 0.5. DPN^+ – 0.15 μ moles. Sample composition: 2 ml glycine buffer, pH 10 + 1 ml water + 0.1 ml lactate + 0.1 ml DPN^+ + 0.02 ml lactic dehydrogenase.

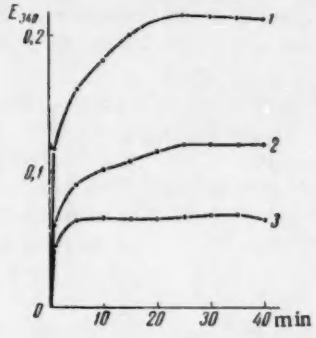


Fig. 5. The effect of DPN^+ concentration on the lactic dehydrogenase reaction. Sample composition: lactate + DPN^+ + lactic dehydrogenase. DPN^+ content of samples in μ moles: 1) 0.15; 2) 0.075; 3) 0.037; lactate – – 50 μ moles. Remaining components as in fig. 4.

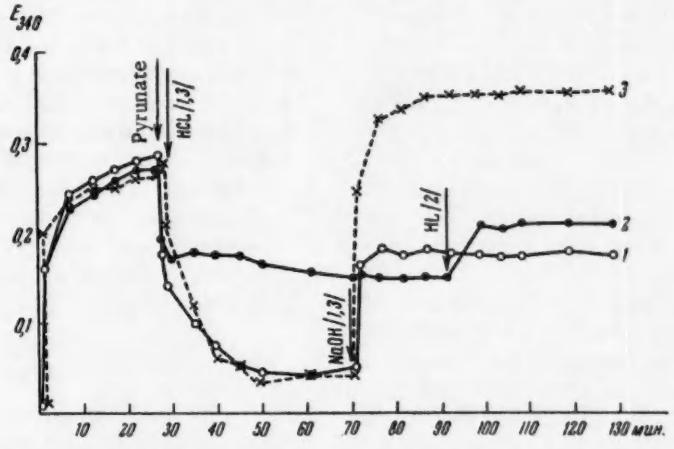


Fig. 6. The effect of pyruvate and pH on the direction and rate of the lactic dehydrogenase reaction. To the starting samples (lactate + DPN^+ + + lactic dehydrogenase) were added in succession: 1) Pyruvate, HCl, NaOh; 2) pyruvate, lactate; 3) HCl, NaOh. Amounts added: pyruvate – – 0.05 ml, 0.05 M; lactate – 0.1 ml, 0.5M; HCl – 0.04 ml, 1M; NaOH – – 0.02 ml, 2M. Remaining components as in Fig. 4.

reduction and oxidation of DPNH to DPN^+. Under these conditions, an increase in pyruvate was not obligatory for reversal of the reaction, as was an increase in the hydrogen ion concentration

DISCUSSION

The data of this study make it possible to characterize roughly the rates of dehydrogenase reactions involved in the glycolytic breakdown of carbohydrates.

In these experiments the α-glycerophosphate dehydrogenase reaction turned out to be especially rapid. In a 1-ml sample, more than 1.0 μ mole of DPNH could be oxidized to DPN^+ in a few seconds. Oxidation of phosphoglyceraldehyde was less rapid.

In samples containing ~ 3.0 μ moles of phosphotriose, 1.4 μ moles of DPN^+, and 0.3-0.7 mg of triose phos-

phate dehydrogenase, ~0.3-0.4 μ mole of DPNH was formed during a 30-60 minute incubation period at room temperature. The reaction ceased at a DPN^+/DPNH ratio of ~ 3.0

In a reaction mixture containing 5 μ moles of lactate, 0.15 μ mole of DPN^+, and 0.02 mg of a lactic dehydrogenase preparation, ~0.06 μ mole of DPNH was formed before the establishment of an equilibrium. The DPN^+/DPNH ratio was ~1.5. This ratio depended on lactate concentration and DPN^+ concentration. At low DPN^+ concentrations, the coenzyme was the factor limiting the reaction rate.

In all the dehydrogenase reactions, equilibrium was established at a point at which DPN^+ exceeded DPNH. These results are in agreement with published information [24]. DPN^+ is the predominant form in animal tissues as well, exceeding DPNH two or three times [25]. Holzer, Schulz, and Lynen [26] found considerably higher DPN^+/DPNH ratios in yeast. Under anaerobic conditions, the DPN^+ concentration was 400 times higher than that of DPNH.

The rapid reaction between DPNH and dihydroxyacetone phosphate, which is catalyzed by an α-glycerophosphate dehydrogenase associated with aldolase, is of interest in connection with the mechanism of regulation of amounts of oxidized and reduced diphosphopyridine nucleotides in animal cells. This enzyme, which has been studied in isolated form by a number of workers [27-30], is a soluble enzyme from sarcoplasm. In mammalian muscle it is more active than the α-glycerophosphate dehydrogenase associated with mitochondria which acts in conjunction with the cytochrome system [30].

The soluble α-glycerophosphate dehydrogenase is coupled through the DPN^+-DPNH system to the 3-phosphoglyceraldehyde dehydrogenase. The relative rates of the reactions catalyzed by these enzymes determine the DPN^+/DPNH ratio of the reaction medium. This is strikingly shown in an enzyme system composed of fructose-1,6-diphosphate, DPNH, and the three enzymes which catalyze the successive reactions: 1) phosphotriose formation (aldolase); 2) dihydroxyacetone phosphate reduction (soluble α-glycerophosphate dehydrogenase); 3) 3-phosphoglyceraldehyde oxidation (3-phosphoglyceraldehyde dehydrogenase).

DPNH, which is oxidized in the α-glycerophosphate dehydrogenase reaction and then reduced in the triose phosphate dehydrogenase reaction, is once more oxidized in the presence of dihydroxyacetone phosphate by α-glycerophosphate dehydrogenase. As shown by these experiments, even in the presence of arsenate, which renders phosphoglyceraldehyde oxidation irreversible [23], the formation of 3-phosphoglyceric acid may go to completion without accumulation of DPNH.

Under these experimental conditions, only when the α-glycerophosphate dehydrogenase was inactivated after having acted on dihydroxyacetone phosphate (by heating 1 minute at 100°) could the oxidation of phosphoglyceraldehyde be detected by an increase in the extinction at 340 mμ.

In animal cells there are various hydrogen acceptors for the DPNH→DPN^+ reaction. In addition to dihydroxyacetone phosphate, pyruvate may act as an acceptor under anaerobic conditions, with α-glycerophosphate dehydrogenase and lactic dehydrogenase competing for the available DPNH. The outcome of such competition, which is affected by properties, as yet little studied, of the interaction of the DPN^+−DPNH system with the enzymes and their substrates, apparently determines whether the carbohydrates of the cells are converted into lactic acid or into glycerophosphate which is then utilized in phospholipid synthesis. The interaction of α-glycerophosphate dehydrogenase and 3-phosphoglyceraldehyde dehydrogenase exerts an influence on the DPNH level, and, through this, on the lactic dehydrogenase reaction, the final reaction of glycolysis, as well. The predominance of DPN^+ in animal tissues is also, apparently, a consequence of the interaction of these enzymes.

When substrates, coenzymes, and enzymes were employed in low concentrations, an increase in fructose--1,6-diphosphate, phosphotrioses, DPN^+, or DPNH invariably led to an increase in reaction rate.

In accordance with this, it may be supposed that a change in the concentration of substrates and coenzymes plays a prominent role in the alteration of rates of enzymatic reactions which occurs during fuctional shifts in carbohydrate metabolism in animal tissues. It seems less probable that such alterations are due to an instantaneous formation or disruption of apoenzyme moieties.

Further studies should make it possible to evaluate more accurately the quantitative aspects of the relationship between the individual dehydrogenases, the DPN^+−DPNH system, and the substrates which arises during the anaerobic breakdown of carbohydrates.

SUMMARY

In anaerobic carbohydrate metabolism the dehydrogenase reactions lead to high level of DPN^+ concentration at the expense of DPNH.

In experiments with low substrate concentrations the ratio DPN^+/DPNH during lactic acid dehydrogenase and triose phosphate dehydrogenase reactions reached 1.5−3.0 and was still higher in the course of the α-glycerophosphate dehydrogenase reaction.

When aldolase, α-glycerophosphate dehydrogenase and triose phosphate dehydrogenase were added in consecutive order the oxidation of 3-phosphoglyceric aldehyde was not accompanied by accumulation of DPNH. DPNH of the reaction of triose phosphate oxidation was used for reduction phosphodioxyacetone with formation of DPN^+. The elimination of α-glycerophosphate dehydrogenase from this system led to DPNH

accumulation proportional to the rate of 3-phosphoglyceric aldehyde oxidation.

During glycolysis rates of oxidation of α-glycerophosphate, triose phosphate and lactic acid by adequate dehydrogenase determine the DPN^+/DPNH ratio usually with predominance of the oxidative form.

Apparently the ratio DPN^+/DPNH is of some importance for regulation of rates of dehydrogenase reactions associated with functional changes of carbohydrate metabolism.

LITERATURE CITED

[1] V.A. Éngel'gardt, Abstracts of the Proceedings of the VIIth All-Union Congress of Physiologists [in Russian] (Izd. AN SSSR, Moscow, 1955), p.700.

[2] A.I. Kolotilova, Biokhimiya 10,423 (1945).

[3] A.I. Kolotilova, Vestn. Leningr. Un-ta No. 7, 49 (1952).

[4] A.I. Kolotilova, Usp. Sovremennoi Biol. 43, 12 (1957).

[5] S.A. Neifakh and M.P. Mel'nikova, Biokhimiya 23, 440 (1958).**

[6] S.A. Neifakh and M.P. Fomina, Biokhimiya 22, 476 (1957).**

[7] S.A. Neifakh, M.P. Mel'nikova, and F.V. Mozhaiko, Doklady Akad. Nauk SSSR 91, 557 (1953).

[8] M.P. Mel'nikova and S.A. Neifakh, Biokhimiya 19, 425 (1954).

[9] G.E. Vladimirov, Uch. Zap. LGU 164, 328(1954).

[10] N.P. Meshkova and S.E. Severin, Manual of Animal Biochemistry [in Russian] (ISN, Moscow, 1950),p. 26.

[11] G. Cori, M. Slein, and C. Cori, J. Biol. Chem. 173, 605 (1948).

[12] W.B. Dandliker and J.B. Fox, J. Biol. Chem. 214, 275 (1955).

[13] I.F. Taylor, A.A. Green, and G.T. Cori, J. Biol. Chem. 173, 591 (1948).

[14] E. Racker, J. Biol. Chem. 167, 843 (1947).

[15] H.G. Albaum, A. Hirshfeld,and A.E. Sobel, Proc. Soc. Exptl. Biol. and Med. 79, 682 (1952).

[16] E.C. Slater, Biochem. J. 53, 157 (1953).

[17] M.F. Gulyi, Abstracts of the Proceedings of the IXth Congress of the Society of Physiologists, Biochemists, and Pharmacologists [in Russian] (Izd. AN SSSR, Minsk, 1959), p. 96.

[18] J.B. Neilands, Methods in Enzymology 1, (Acad. Press Publ., New Jersey, 1955) p. 449.

[19] I.F. Taylor, S.F. Velick, and G.T. Cori, et al., J. Biol. Chem. 173, 619 (1948).

[20] B.L. Horecker and A. Kornberg, J. Biol. Chem. 175, 385 (1948).

[21] I. Secuzu, B. Hagihara, and F. Hattori, et al., J. Biochem. 44, 587 (1957).

[22] J.B. Fox and W.B. Dandliker, J. Biol. Chem. 218, 53 (1956).

[23] O. Warburg and W. Christian, Biochem. Z. 303, 40 (1939).

[24] H.I. Hohorst, Biochem. Z. 328, 509 (1957).

[25] H. Frundler, Z. Physiol. Chem. 297, 267, (1954).

[26] H. Holzer, G. Schulz, and F. Lynen, Biochem. Z. 328, 252 (1956).

[27] H. Euler, E. Adler, and G. Gunther, Z. Physiol. Chem. 249, 1 (1937).

[28] T. Baranowski, J. Biol. Chem. 180, 535 (1949).

[29] N.K. Nagradova, Biokhimiya 24, 336 (1959).**

[30] R.W. Estabrook and B. Sacktor, J. Biol. Chem. 233, 1014 (1958).

**Original Russian pagination. See C.B. Translation.

A COMPARATIVE IMMUNOLOGICAL INVESTIGATION OF NUCLEOPROTEINS ISOLATED FROM REGENERATED FILTERABLE FORMS OF TYPHOID FEVER BACTERIA

A.N. Bitkova and E.I. Koptelova

Biochemistry Department, N. F. Gamelei Institute of Epidemiology and Microbiology, Academy of Medical Sciences USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 417-421, May-June, 1960
Original article submitted August 14, 1959

In a previous publication [1] it was shown that antigenic complexes isolated by TCA from typhoid fever culture and from regenerated "secondary" cultures No. 9 "sk" and No. 3 differed somewhat in their chemical composition. "Secondary" cultures No. 9 "sk" and No. 3 were isolated from the initial typhoid fever culture "A", which was typical in its biological properties. Culture No. 9 "sk" was identical to the initial typhoid fever culture in its biological properties and differed only in incomplete restoration of the original degree of virulency. Culture No. 3 was profoundly different from the initial culture it was inert in enzymatic and serological respects; its antigenic properties and virulency were lowered.

In relation to this it was of interest to compare other microbial cell components, such as nucleoproteins and nucleic acids. Since the nucleoprotein content of the bacterial cell is quite high [2], the study of nucleoprotein composition of these cultures and their content of nucleic acids became of definite interest.

In the present study the following problem was presented: to study the qualitative and quantitative changes which could take place in nucleoproteins isolated from cultures differing in biochemical, antigenic and virulent properties.

METHODS AND RESULTS

As the material for investigation there was utilized acetone-extracted fat-free microbial bodies, obtained from the initial typhoid fever culture "A" and from two "secondary" regenerated filterable form cultures No. 9 "sk" and No. 3.

Nucleoproteins from these cultures were isolated by the method of Belozerskii [2] following a preliminary extraction of the antigens with TCA: repeated extraction with 0.2% NaOH and precipitation at the isoelectric point with acetic acid. The nucleoprotein precipitate was separated by centrifugation, washed with water, and dried with alcohol and ether. In the obtained preparation total N content was determined by micro-Kjeldahl total P according to Fiske-Subbarow, various forms of phosphorus, with regards to nucleic acids, according to Schmid and Tannahauser [3], and DNA according to the reaction of Diche with diphenylamine.

Comparison of the obtained data showed that nucleoprotein of the initial typhoid fever culture "A" and of the regenerated culture No. 9 "sk" differed basically from the nucleoprotein from the regenerated culture No. 3 in their content of total P, RNA P and DNA P. Nucleic acid content in the nucleoprotein isolated from regenerated culture No. 3 was two-fold lower than in nucleoprotein obtained from the two other cutltures. The pointed-out differences, evidently, should be taken as a change in nucleotide content of nucleoproteins from culture No. 3, biological properties of which, as was already shown, were considerably altered.

The obtained data are fully in accord with results from the study of the biological properties of the investigated cultures. Thus, the regenerated culture No. 9 "sk", closely related in its chemical composition to typhoid culture "A", was identical with the latter in its biological expressions. The regenerated culture No. 3, which was pronouncedly different in its biological properties from the initial culture and the "secondary" culture No. 9 "sk", was likewise different in its chemical structure.

From Table 1 it is also evident that a large portion of P in the nucleoproteins belonged to nucleic acids. The DNA content in the nucleoprotein of the secondary culture No. 9 was somewhat greater than in the initial culture. Analogous data were obtained by Galkina in 1958 [4].

After separating nucleoproteins into fractions, Belozerskii and coworkers [5-7] have established that bonding of nucleic acid and proteins differed. In one case the nucleic acids were separated from the protein easily, and in other cases they could not be separated from the protein. According to Belozerskii, and this is supported by other authors [8-9], there exist two types of bonds: labile or salt-forming, and a more firm bond between the protein and the nucleic acid. In connection with this it was of interest to clarify whether there were differences in the protein-nucleic acid bonds in nucleoproteins of the regenerated cultures, and especially in

TABLE 1. Nucleoprotein Analysis of Various Cultures in % of Dry Matter

Cultures	Yield	Total N	Total P	Nucleic acid P	RNA	DNA	Total nucleic acids as P
Typhoid fever "A"	5—7	11,94	4,60	4,28	25,29	17,77	43,06
Regenerated filterable form No. 9 "sk"	5—7	11,45	4,25	3,97	21,43	19,89	41,32
Regenerated filterable form No. 3	6—8	10,78	2,35	2,14	11,10	8,99	21,66

TABLE 2. Chemical Composition of Nucleoprotein in Fractions of Different Cultures

Nucleoprotein fraction	Culture	Weight, g	Total N	Total P
			in % of absolute dry weight	
Protein B	"A"	0,391	15,90	0,12
	No.9 "sk"	0,387	15,01	0,21
	No.3	0,441	14,23	0,15
Acid salt of nucleoprotein-V and nucleic acid -D	"A"	0,302	—	6,80
	No.9 "sk"	0,268	—	6,77
	No.3	0,183	—	6,75
V - true nucleoprotein (thymonucleic acid, firmly bound with the protein)	"A"	0,105	14,75	4,42
	No.9 "sk"	0,104	13,74	3,66
	No.3	0,098	12,27	2,12
D -Nucleic acid	"A"	0,189	15,03	8,69
	No.9 "sk"	0,155	14,54	8,45
	No 3	0,055	12,25	8,58

nucleoproteins of culture No. 3 in comparison with the initial typhoid culture.

Nucleoprotein fractionation. Nucleoprotein fractionation into separate components was conducted according to the method worked out by Belozerskii [2].

One gram of dry nucleoproteins (fraction A) was dispersed in cold with distilled water and then dissolved in 5% ammonia. Concentration of nucleoproteins in the solution was ~2%. The obtained clear solutions were acidified with acetic acid to a point of slight clouding of the solution. In order to precipitate the proteins, there was added to the solutions, with strong mixing, a saturated solution of picric acid; in order to obtain complete precipitation of the protein picrates (fraction B), the solution was acidified with acetic acid. The formed precipitate was separated by centrifugation, washed with water and then with alcohol and by means of reprecipitation it was freed from picric acid. To the filtrates there was added 1.5 volumes of ethanol and the mixture was acidified with HCl. Into the precipitate fell compounds rich in phosphorus, which were composed of nucleoproteins (fraction V) and nucleic acids (fraction D). In order to separate the latter the precipitate was dissolved with a weak alkaline solution and to it was added excess of acetic acid. The obtained precipitates, "true" nucleoproteins (fraction V) were washed with water, alcohol and dried in a vacuum dessicator. Nucleic acids, D, were isolated from the filtrate by adding to it 1.5 volumes of ethanol and then HCl until a positive Congo paper reaction appeared. In order to purify the nucleoprotein fraction from foreign admixtures it was redissolved and reprecipitated a number of times.

In this way, as the result of fractionation, all nucleoproteins were subdivided into fractions: protein B, acid salt of nucleoprotein and nucleic acids with a raised (up to 6%) phosphorus content V + D, true nucleoprotein V and nucleic acid D. Nucloprotein fractionation data from the different cultures are presented in Table 2.

The nucleoproteins did not differ qualitatively in the character of protein-nucleic acid bonds. With regards to the total yield of fractionsV and D and fractions V and D separately, the greatest yield was obtained during breakup of nucleoprotein from the initial typhoid fever culture; the smallest yield , from fractionation of nucleoprotein from culture No. 3. Protein B and "true" nucleoprotein V gave positive qualitative Biuret reaction. Fraction D was intensly stained with orcein for RNA; the same reaction with "true" nucleoproteins resulted in very weak staining. Qualitative tests for DNA with diphenylamine produced the opposite result, brighter staining with "true" nucleoprotein (fraction V) and a very weak -- with nucleic acid (fraction D). A positive Feulgen reaction was obtained only with true nucleoprotein. The last reaction confirmed the presence of DNA in the "true nucleoprotein". Aside from that, from the obtained data it is evident that into the composition of the investigated nucleoproteins enter proteins which can be joined with nucleic acids in a "labile" or firm form. At the same time, a large protion of the protein in nu-

cleoproteins was joined with nucleic acids in a "labile" form. Attempts to separate "true" nucleoprotein V into protein and nucleic acid with the help of picric acid did nto produce the desired results. This pointed to the firmness of the bond between the protein and nucleic acids.

In order to conduct qualitative comparisons of the composition of nucleoproteins studied, the method of paper partition chromatography was utilized with two solvents: butyl alcohol - acetic acid - water in the ratios of 40:10:50 or 40:15:45. The solvents were let through the chromatograms stepwise. In the nucleoprotein hydrolysate there were discovered 19 amino acids: cysteine, lysine, histidine, arginine, aspartic acid, serine, glycine, hydroxyproline, glutaric acid, threonine, α-alanine, proline, tyrosine, α-aminobutyric acid, methionine, valine, phenylalanine, isoleucine and leucine. Proline and hydroxyproline were developed by a special reaction with isatin [10]. Qualitative chromatographic analysis had revealed that the amino acid composition of the protein component from nucleoproteins studied was the same.

In order to compare the antigenic properties of the nucleoproteins studied, rabbits were immunized and antisera were obtained these were utilized for the ring-precipitation reaction. The conducted investigation allowed us to clarify not only the similarites of the antigenic properties of nucleoproteins from the initial and regenerated cultures, but also the appearance in these latter of specific antigenic components. Adsorption of the precipitins was conducted in order to determine more accurately the peculiarites of the antigen structure of regenerated cultures and to compare it with the initial. For this purpose to 1 ml of immune serum there was added 1 ml of nucleoprotein solution. The mixture was held at 37° for four hours, then at 4° for 18-24 hrs and then it was centrifuged. The supernatant was utilized for the precipitation. The ring-precipitation reaction with nucleoproteins of the various cultures gave the following results:

Nucleoproteins	Antisera to cultures "A"	No. 9 "sk"	No. 3
Typhoid fever culture "A"	1 : 100	1 : 10	1 : 10
Secondary culture No. 9 "sk"	1 : 10	1 : 100	1 : 10
Secondary culture No. 3	1 : 10	1 : 10	1 : 100

The presence of specific components was confirmed by supplementary adsorption of precipitins in uncleoprotein antisera and with a subsequent ring-precipitation reaction. Separate portions of antisera against nucleoproteins 9 "sk" were exhausted with nucleoproteins from cultures "A" and No. 3, antisera against nucleoprotein No. 3 were exhausted with nucleoproteins "A" and No. 9 "sk", and then a cross-precipitation reaction was conducted. With the different nucleoproteins the following results were obtained:

Antisera against nucleoproteins	Adsorbed by nucleoproteins	"A"			No. 9 "sk"			No. 3		
		1 : 5	1 : 10	1 : 100	1 : 5	1 : 10	1 : 100	1 : 5	1 : 10	1 : 100
No. 9 "sk"	"A"	—	—	—	+	+	—	+	+	—
No. 9 "sk"	No. 3	+	—	—	+	+	—	—	—	—
No. 3	"A"	—	—	—	+	+	—	+	+	—
No. 3	No. 9 "sk"	—	—	—	—	—	—	+	+	—

As is evident from the data, during exhaustion of antiserum against nucleoprotein No. 9 "sk" with nucleoprotein "A" there was complete extraction of antibodies to nucleoprotein "A", and there were left antibodies to nucleoproteins No. 9 "sk" and No. 3. During exhaustion of the given antiserum with nucleoprotein No. 3, in the antiserum there were observed antibodies against nucleoprotein "A". During exhaustion of nucleoprotein No. 3 antiserum with nucleoprotein "A" there was complete extraction from the antiserum of antibodies against nucleoprotein "A" and appearance of nucleoprotein No. 9 "sk" and homologous antibodies. During exhaustion of the given nucleoprotein No. 9 "sk" antiserum there were observed only antibodies against nucleoprotein No. 3. In this way, the study of nucleoproteins calls attention to the presence in "secondary" cultures of specific together with group antigens.

Numerous replications of these experiments were always accompanied by the same results, attesting to the fact that in the process of regeneration of "secondary"

cultures from filterable forms, in parallel with the restoration of cultural, biochemical and antigenic properties of the initial culture, there occurred restoration of common antigenic components of the full antigen at the expense of nucleoproteins of the initial culture.

The specific antigenic structure of the regenerated filterable form cultures depended on the formation of specific antigenic properties of the nucleoproteins.

SUMMARY

Nucleoproteins isolated from the initial typhoid fever culture "A" and from regenerated filterable form, No. 9 "sk", differed from nucleoprotein obtained from regenerated "secondary" culture No. 3 in their content of total phosphorus and nucleic acids. The content of the latter in nucleoprotein isolated from regenerated culture No. 3 was one half of the content in the other two.

Fractionation of the nucleoproteins studied showed that they were not individual compounds but could be subdivided into fractions: protein, firm complex of protein and nucleic acid, and nucleic acid. A large portion of the protein in nucleoproteins was not firmly bound.

Paper partition chromatography did not reveal qualitative differences in the amino acid composition of the protein component of the nucleoproteins studied.

During the study of the antigenic properties of nucleoproteins from regenerated filterable form bacteria there were revealed specific antigens along with group antigens.

LITERATURE CITED

[1] A.N. Bitkova, G.Ya. Kagan, M.A. Atabegova and E.I. Koptelova, in: Transformation of Microorganisms, Vol. 2. [in Russian] (AMN SSSR, Moscow, 1957), p. 301.

[2] A.N. Belozerskii and N.I. Proskuryaev, Practical Procedures in Plant Biochemisty [in Russian] ("Sov. Nauke", Moscow, 1951), p. 212.

[3] G. Schmid and S. Tannahauser, J. Biol. Chem. 161, 1, (1945).

[4] G.O. Galkina, Microbiol. J. 20, 9 (1958).

[5] A.N. Belozerskii, Biokhimiya, 1, 254 (1956).*

[6] A.N. Belozerskii and L.A. Chernomordikova, Biokhimiya 5, 133 (1940).

[7] A.N. Belozerskii and S.O. Uryson, Biokhimiya 25, 568 (1958).*

[8] S.E. Manoilov, B.A. Orlov and O.N. Setkina, Biokhimiya 13, 337 (1948).

[9] S.E. Manoilov and A.S. Orlov, Biokhimiya 23, 663 (1958).*

[10] R. Acher, C. Fromageot and M. Jutisz. Biochim. et biophys. acta 5, 81 (1950).

*Original Russian pagination. See C.B. Translation.

THE QUESTION OF THE BINDING OF LABELED METHIONINE-S^{35} BY PROTEINS

M.S. Volkova, L.V. Komarova, and A.G. Pasynskii

A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR, Moscow, and Yaroslav Medical Institute

Translated from Biokhimiya Vol. 25, No. 3, pp. 422-426, May-June, 1960
Original article submitted August 15, 1959

Investigations of the binding of labeled methionine-S^{35} by pure proteins in the organism have been widely used in biochemistry for study of various metabolic processes. Recently the specific activities of labeled methionine preparations have reached very high values (up to 1 curie/g) which has made the technique of measurement easier. However, in a number of studies [1-4] it has been shown that under the action of ionizing radiation on methionine solutions there is marked degradation with formation of a series of decomposition products. For example, as a result of the action of β- and γ-rays [1] on dilute solutions of pure methionine, up to eight different substances have been found, including products of oxidation of methionine (to sulfoxides and sulfones) and its splitting with rupture of the C–S bond.

Kolousek and coworkers [2-4] have shown that under the action of x-rays and β-rays on methionine solutions many degradation products of methionine are also found. The authors have showed that in preparations of methionine with high specific activity similar oxidation or decomposition of methionine can occur as a result of internal self-irradiation by β-rays. Therefore, in work with such methionine preparations or when they are kept, a large number of substances accumulate in them which have various chemical properties. There is no doubt that this fact must be considered in all investigations using labeled methionine. The study of this question with regard to binding of labeled methionine by proteins is the object of our work.

EXPERIMENTAL

The experiments were carried out in 1957-1959. For the study we used preparations of methionine-S^{35} with the following specific activities: preparation No. 1, 145 mcurie/g, preparation No. 2, 637 mcurie/g, and preparation No. 3, 300 mcurie/g. The preparations were submitted to paper chromatography on paper (Leningrad "slow") first treated with a 0.4% solution of alcoholic alkali and an 0.8% HCl solution and carefully washed with distilled water to disappearance of Cl ion. The labeled methionine was placed on the paper as a narrow strip in the amount of 100 to 300 μg.

As solvent we used a mixture of butanol saturated with water and 85% formic acid in the ratio 9:1. Chromatography was continued for about a day. The chromatogram, dried at room temperature, was submitted to radioautography on film of type XX or X-5. After development, the film showed from eight to twelve fractions. The corresponding spots on the chromatogram were cut out, eluted with water, and after filtration the radioactivity was measured in each extract.

In measuring the adsorption of the fractions by protein (serum albumin) we prepared five to seven solutions of serum albumin (with the methionine fraction preparations) were denatured by the method described previously [5, 6] using x-rays (3BPM-200 tube, 180 kv, 15 ma, dose 20,000 r) or by heating on a water bath for 25 min at 80°. After denaturation the protein was precipitated by addition of an equal volume of 10 or 20% TCA. The precipitate was washed with a mixture of borate buffer, pH 7.8 and 10% TCA in the ratio 1:1 with repeated centrifuging (20 times) or on a filter up to 80 times to absence of activity in the evaporated wash water. In this way we determined the fraction of methionine bound by protein at different concentrations of fraction in solution, that is, the adsorption isotherm of the fraction.

In some of the experiments we also measured the decreased activity of the precipitate after washing the bound fraction with a solution of unlabeled methionine. For this purpose, a part of each precipitate was placed on a target, dried to constant weight (at 100-110° for 7-8 hours) and the activity was checked with an end counter. Another part of each precipitate was dissolved in several drops of 2 N NaOH and the solution was treated with 3 mg of unlabeled methionine; after standing for several hours the protein was again precipitated by adding an equal volume of 20% TCA and the activity of the precipitate was again determined.

RESULTS

Chromatography of the methionine preparation showed the presence in it of 8 to 12 fractions (Fig. 1). Comparison of the pure unlabeled methionine which gives only one ninhydrin-positive spot on the chromatogram with the labeled acid showed that only fraction 6 corresponded to methionine itself. The spots of the other fractions appeared in the labeled methionine pre-

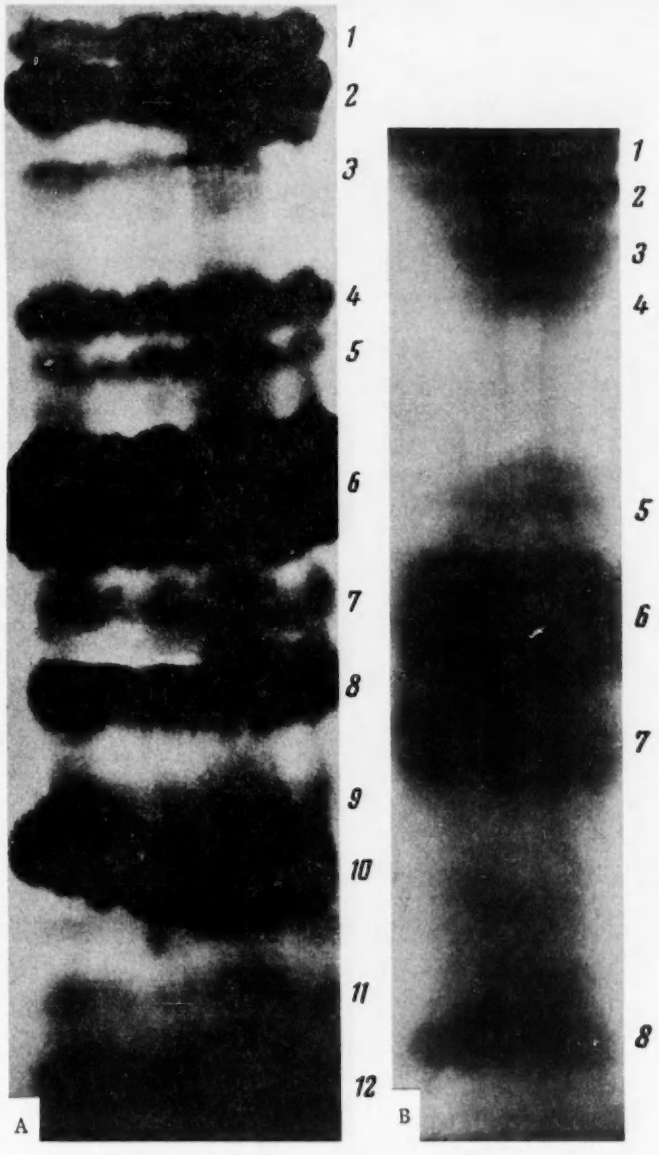


Fig. 1. Radioautogram of methionine and its degradation products. A) Preparation No. 2; B) preparation No. 3; figures denote the number of fractions. Fraction 6 corresponds to pure methionine.

parations even on the first day after synthesis of the preparations (we tested this for laboratory and prepared samples); when the preparations were kept, the intensities of the different fractions changed. Figure 1 shows that the content of the various fractions in different preparations is very different, especially for preparation No. 2 in which the methionine content (fraction 6) has reached 83%; the relative content of the different fractions is shown in the table.

According to the adsorption of the different fractions on heat-denatured serum albumin, they differ considerably among themselves (Figs. 2 and 3). Figure 2 shows that the adsorbability of fractions 1 and 2 greatly surpasses the adsorption of pure methionine (fraction 6). In Fig. 3 the adsorbability of fraction 2 is higher than for fractions 5 and 6, which are close to each other, while the adsorbability of fraction 1 is almost twice as great as that of the other fractions. Figure 2 shows also that the

Quantitative Content of Fractions in Preparation No. 2

Fraction No.	Counts in 5 ml solution	% of total counts
1	15750	2.04
2	43125	5.6
3	6370	0.83
4	16000	2.07
5	27100	3.52
6	640850	83.16
7	5900	0.76
8	5810	0.77
9	1450	0.19
10	3100	0.40
11	1200	0.15
12	3950	0.51

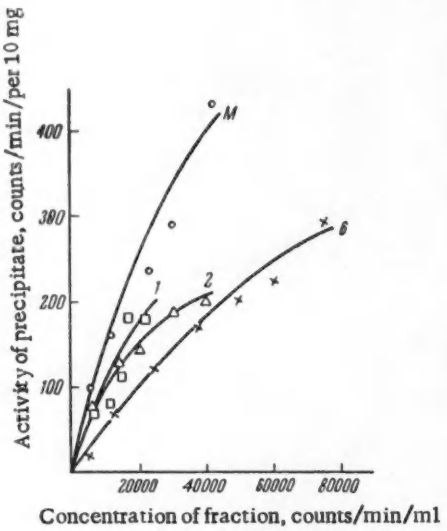


Fig. 2. Adsorption isotherms of fractions 1, 2, and 6 and unfractionated preparation (M) No. 2 of labeled methionine on serum albumin

activity of the bound, labeled, unfractionated methionine has almost twice the adsorbability of fraction 6, although the content of the latter is about 85%; actually, the same conclusion follows from Fig. 3 where the activity in bound fraction 1 is twice as high as in fraction 6 in comparing the number of counts in solution.

The different behavior of the fractions is also evident in the experiments on desorption of protein-bound fractions by solutions of unlabeled methioine. Thus, for example, the amount of binding of the different fractions in Fig. 2 is decreased after treatment of the precipitate by unlabeled methionine for fraction 1 by 40%, for fraction 6 and for unfractionated methionine by 50%. Comparison of these data with the adsorption isotherms in Figure 2 clearly shows the presence in methionine-S^{35} of small amounts of strongly adsorbing admixtures which are correspondingly weakly washed out by solutions of unlabeled methionine.

In denaturation of the serum albumin by irradiation with a dose of 20,000 r (at a methionine content of 40,000 counts /min/ml of mixture), the relative increase of binding as compared with unirradiated protein for pure methionine (preparation No. 3, fraction 6) was about five-fold, while for fractions 1 and 7 it was 1.8-fold, for fractions 2 and 4, 2.6-fold, for fraction 5, 2.0-fold, and for fractions 3 and 8, 3.0-fold. Thus for different preparations of labeled methionine and different methods of denaturation of the protein, the fractions behave in very different ways.

DISCUSSION OF RESULTS

The above data, along with the results of Kolousek and coworkers [2-4], show the presence of internal radiochemical degradation of methionine as a result of its self-irradiation by β-rays which for preparations with high specific activity is already high, soon after the synthesis. As a result, the preparations usually contain 8-12 fractions of which some give the ninhydrin reaction and some are shown only by radioautographs. The quantitative distribution of the fractions for preparation No. 2 is shown in the table, from which it is clear that most of the fractions occur in very small amounts; this makes it especially difficult to isolate the fractions and identify them chemically. According to the data of Kolousek et al. [2], the methionine preparations contain products of demethylation, deamination, and oxidation of methionine, particularly methionine sulfoxide, homo-

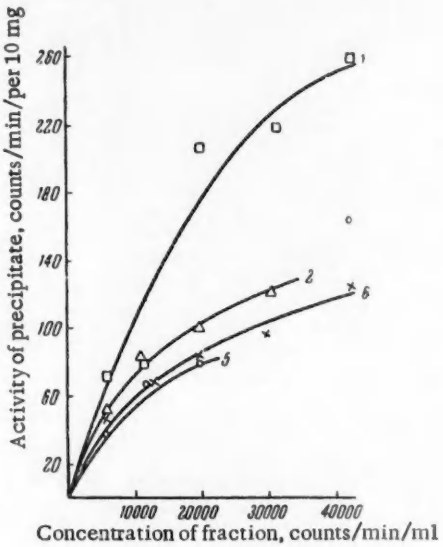


Fig. 3. Adsorption isotherms of fractions 1, 2, 5, and 6 of preparation No. 1 for methionine-S^{35} on serum albumin.

cysteic acid, etc. Comparison of the Kolousek chromatograms with our results (Table) show that fraction 1 corresponds to homocysteine, fraction 2 to methionine sulfoxide, fraction 3 to methionine sulfone, fraction 6 to pure methionine, but most of the fractions remain unidentified. However, while our preparation No. 2 (Table) contains fraction 6 in the amount of 83.16%, for Kolousek there was only 67%; the content of fraction 2 in our preparation was 5.6% and for Kolousek, 20%; the content of fraction 1 in our preparation was 2.04%, and for Kolousek, 5.2%. These differences are evidently explained by the fact that the preparation of Kolousek, obtained from the USSR with an activity of 800 mcurie/g, had undergone a longer period of destruction.

Kumta et al. [1] suggested the presence in irradiated preparations of methionine of amino acids with R_f values corresponding to leucine, isoleucine, aspartic acid, aminobutyric acid, etc.

Actually, in studying the binding of labeled methionine by proteins or by animal organs, the various fractions are adsorbed differently and therefore in estimating the distribution of methionine by the residual activity of the precipitate without taking account of the numerous components of the methionine preparations we can reach inaccurate conclusions. Thus, for example it follows from Fig. 2 that with an activity of the solution of 20,000 counts /min/ ml of mixture the total binding of unfractionated preparation (M) per 10 mg of precipitate is about 240 counts, of which the methionine itself (fraction 6) makes up 83% of the preparation (that is, 16,600 counts) which comes to 80 counts only, and the rest of the activity is due to binding of other substances by the protein. For our earlier methodof observing the denaturative changes of proteins in binding labeled methionine [5, 6] this fact was not significant, since our measurements were only the total increase in activity of the protein precipitate as a qualitative indication of the presence of denaturation. In a more detailed biochemical investigation we must consider that the observed changes in activity may be due to products of methionine decomposition. Therefore we must especially avoid use of desorption of the precipitate by unlabeled methionine to establish the amount of chemically bound methionine, since in this treatment substances may remain in the precipitate which are not washed away by methionine and which have an entirely different chemical nature.

It is also necessary to use care in interpreting the presence of a series of spots on the methionine chromatogram in a biochemical investigation of its metabolism in the organism as the result of enzymatic transformation of methionine into other substances. In neglecting the radiochemical destruction of labeled methionine and the rapidity of this process there may be sources of error in the biochemical study (for example, it may be incorrect to draw conclusions as to the transformation of methionine into homocysteic acid, etc.). In order to decrease these errors we must work with chromatographically separated fractions of pure methionine under conditions of use of these fractions immediately after isolation. It is possible that analogous facts may apply also in work with preparations of other amino acids or other compounds (such as NaI^{131} [8]) with high specific radioactivity.

SUMMARY

In preparations of labeled methionine-S^{35} with high specific radioactivity, as a result of internal self-radiation, there may be a large number of products of its degradation or oxidation (up to 8-12 fractions) which differ sharply in combining with proteins and in desorption by unlabeled methionine; this must be considered in all biochemical investigations with such preparations.

LITERATURE CITED

[1] U. Kumta, S. Gurnani, and M. Sahasrabudhe, J. Sci. Ind. Res. 15, 45 (1956).

[2] J. Kolousek, J. Liebster and A. Babicky, Nature 179, 521 (1957).

[3] J. Kopoldova, J. Kolousek, and A. Babicky, Nature 182, 1074 (1958).

[4] J. Kolousek, J. Liebster, and A. Babicky, Coll. Czech. Chem. Comm. 22, 874 (1957).

[5] A.G. Pasynskii, M.S. Volkova, and V.P. Blokhina, Doklady Akad. Nauk SSSR 101, 317 (1955).

[6] T.E. Pavlovskaya, M.S. Volkova, and A.G. Pasynskii, Doklady Akad. Nauk SSSR 101, 723 (1955).

[7] T.I. Kazantseva, in: The Struggle with Silicosis, Vol. 4. [in Russian] (Izd. AN SSSR, Moscow, 1959), p. 117.

[8] V. Doctor and J. Trunnell, Endocrinology 64, 455 (1959).

BIOCHEMICAL CHANGES IN THE ORGANISM OF THE CARP PRODUCED BY ADMINSTRATION OF ANTIGENS

Ya.A. Epshtein, B.G. Avtikyan, N.E. Lavrovskaya,
V.M. Rogozhnikova, and A.G. Artemova

Research Institute of Lake and River Fisheries, State Planning Commision RSFSR, and Institute of Experimental Medicine, Academy of Medical Sciences USSR, Leningrad
Translated from Biokhimiya Vol. 25, No. 3, pp. 427-435,
May-June, 1960
Original article submitted August 17, 1959

The question of the origin of blood plasma proteins and the closely connected question of the genesis of specific antibodies are at present subjects of careful investigation. The existing literature is chiefly concerned with mammals. There is very little information on plasma proteins in cold-blooded animals, especially in fish. It is not clear whether fish can produce specific antibodies in response to antigen stimulation.

We have been unable to find on paper electrophoretograms of blood serum of rainbow trout (Salmo trutta iridens), German carp (Carassius carassius [1]) and carp (Cyprinus carpio) separate protein fractions which correspond in mobility to the γ-globulins of mammals.*

In the recent comparative immunological investigation of Avetikyan [2] it was shown that administration of antigen to the groundling (Misgurnus fossilis) and the German carp (Carassius carassius) does not produce antibodies.

The biochemical and immological studies carried out on fish confirm each other since specific antibodies, as is known, are found among the globulins of blood serum.

Our results agree with those in the literature on the electrophoretic separation of serum proteins in fish [3-5]. At the same time, we should mention that some authors have observed γ-globulins in the serum of fresh water fish [6,7] and other investigators have shown that fish have an inherent function of producting specific antibodies [8,9]. However, such a function appears in certain types of fish, and only under definite conditions [10]. A small amount of γ-globulin can be found in the serum of those marine bony fishes which have plasma cells [4]. In fresh-water fish both γ-globulin and plasma cells are absent from the blood (as is known, these cells play a definite part in immunogenesis in mammals).

These results show the need for parallel investigations of blood serum proteins of fish and their reaction to parenteral injection of antigens. The present paper is devoted to this topic.

MATERIALS AND METHODS

The experiments were carried out on hybrid carp (third generation from Kursk and Amur carp) grown at the experimental station of the Institute of Lake and River Fisheries in Ropesh, the autumn (September-October) and spring(April-May) before spawning. The experimental and control fish were kept under the same conditions in an enamel tank with running water at 6-7° (autumn) and 11-12° (spring). In the autumn the experimental fish were not fed; in the spring they were fed twice a week.

As the antigen we used normal horse serum (HS) and a 10% suspension of washed sheep erythrocytes (SE). The antigen was injected into the dorsal muscle. HS was used in varying doses from 0.005 ml to 0.01 ml/g of the carp at each injection.

The plan of immunization was also varied. In the autumn, HS was injected at three-day intervals ten times; in the spring, three times. The serum of fish which had received HS was studied in experiments with precipitation and prolonged complement fixation at 0°. The SE suspension was injected daily in increasing doses (from 0.1 to 1 ml in each test) for five weeks. Samples of blood taken from the fish of this group were studied after hemolysis.

Blood samples for serological and biochemical investigation were taken at different times (4-30 days) after the end of the cycle of injections of the antigens.

Biochemical analysis of blood serum and liver tissue was carried out on the same day as the immunological analysis. For the analyses the blood from five to seven fish was combined; the blood was taken from the caudal vein after cutting off the tail. No anticoagulant or preservative was added to the blood. The liver was rinsed out with physiological saline through the hepatic vessels, keeping it in melting ice, and was homogenized in the cold in a microgrinder. All the biochemical analyses were carried out immediately after taking the samples.

*At our request, R.Ya. Polyak kindly agreed to check our results, using Whatman paper and the Spinco electrophoretic apparatus, and confirmed our data.

As controls we used carp which had not been injected with antigens.

The total content of serum proteins was determined with a refractometer. Paper electrophoresis (on Leningrad "slow") was carried out with a voltage drop of about 12 v/cm; the phoretogram was stained with bromphenol blue as described by Spinco [11]. Lipids on the electrophoretogram were stained with Sudan black. The lipid content was estimated by the intensity of the color of Sudan black in the serum or protein fraction deposited in a definite volume on the filter paper and dried before the color reaction by the method of Swahn [11]. The photometry was was carried out with an FEK-M electrophotocolorimeter.

We also made an electrophoretic study of the proteins which remained in solution after precipitation by $(NH_4)_2SO_4$, half saturated, and the protein fraction (lipoproteins, LP) precipitated from the supernatant liquid by acidification to pH 3.9 according to Macheboeuf [12]. Electrophoresis was carried out in a veronal buffer with pH 8.6. Phospholipids (PL) were determined either by the amount of fatty acid fraction which did not dissolve in acetone but dissolved in the Bloor alcohol-ether mixture after saponification, or by their phosphorus at the same time as the determination of the other phosphorus fractions. The latter were determined in the blood serum and liver tissue by the method of Schmidt-Thanhauser as modified by Lisovskaya [13] and Yakhina [14] for the determination of phosphoproteins. Lipids were determined from the quantity of fatty acids by conductometric titration [15], running the saponification according to Schmidt-Nielsen [16] without preliminary precipitation of the protein. For this, 0.1-0.2 ml of blood serum or the corresponding amount of Bloor extract from liver tissue was saponified from one to one and a half hours on a water bath at 100° with addition of 1 ml of 1 M KOH. The fatty acids were precipitated with 1 M HCl and removed with ether. After evaporation, the residue was dissolved in 5-6 ml of alcohol and titrated conductometrically with a water solution of 0.01 M alkali (alkali solution gives a less clear determination of the end of titration) using an OKB PEP conductometer. To avoid large blanks, purified and freshly-distilled organic solvents should be used.

Paper electrophoretic separation was carried out with liver proteins dissolved in salt solution according to Kaplanskii [17]. Acid-alkali ratio in the blood serum was determined by the direct method [18]. Residual nitrogen was determined by the Nessler method in protein-free 10% TCA filtrate, amino nitrogen according to Uzbekhov [19], cholesterol according to Neuschloss [20], and liver phosphatase activity according to Bodansky [21].

RESULTS AND DISCUSSION

Until now we have been limited, for the coloring of the serum on the electrophoretogram, to the protein stains, and we have obtained from the number of fractions (but not from the relative content of the separate fractions) a picture resembling that described by Drihlon [3]. Like Drihlon, we tend to consider the first, most mobile fraction as the albumin fraction. In our carp, this fraction consists chiefly of two closely connected fractions: one evenly and weakly colored, distributed in the form of a low plateau ahead of the second, an intensely colored peak. We could not separate these fractions on paper after longer electrophoresis. Beyond this peak at some distance there follows a narrower and less intense peak and beyond this, a third, intensely colored, peak which passes into a weakly colored, gradually falling section. The last part of the electrophoretogram somewhat resembles the section which remains in the paper electrophoresis of albumin. The three clear peaks (Fig. 1) could be designated as the albumin fraction (A), α-globulins, and β-globulins. The final colored section, which has a form more like a plateau or even a small peak, is considered by Drihlon as a γ-globulin fraction. When we stained the electrophoretogram with Sudan black we observed that in the carp blood serum (and also, as we were convinced, in the trout and German carp) only the fraction A was colored especially intensely on its peak and less markedly in the fraction ahead of it. No other fraction in the carp or the other fish which we studied was stained by Sudan black. On the basis of the information in the literature that it is very difficult to separate on paper the LP fraction, which migrates with α_1-globulin, from the albumin fraction, we are inclined to consider the first intensely colored peak stained by both Sudan black and protein reagents as the α_1-globulin fraction with the accompanying LP. In this case, only the part of fraction A ahead of the peak comes from albumins and lipoalbumins. The second, more weakly indicated peak should correspond to the α_2-globulin fraction, and the third to the β-globulin fraction.

Fig. 1. Paper electrophoretog ram of carp serum proteins according to Drihlon (a) and according to our data (b) A) albumin; α_1, α_2, β_1, β_2, γ) the corresponding globulin fractions.

TABLE 1. Total Protein Content and Relative Content of Separate Protein Fractions (in %) in Blood Serum of Controls and Experimental Carp

Period after injecting antigen, days	Protein, %	Albumin	Globulins			
			α_1	α_2	β	γ?
Autumn carp		Control				
	3,5	6	23	20	40	12
	3,2	3	22	24	34	16
		3	26	27	32	12
	3,9	Not determined				
	3,1	Not determined				
	3,6	Not determined				
		6 injections of HS				
6		10	34	21	26	10
20		7	29	22	28	14
Spring carp		Control				
	4,8	6	26	27	28	12
	3,6	Not determined				
	3,3	Not determined				
		3 injections of HS				
7	3,6	14	27	27	24	8
14	3,4	25	38	16	11	10
21	3,2	14	26	20	29	11
26	3,2	16	27		45	13
4	3,5	6	26	25	27	17
10	2,9	11	33		47	9

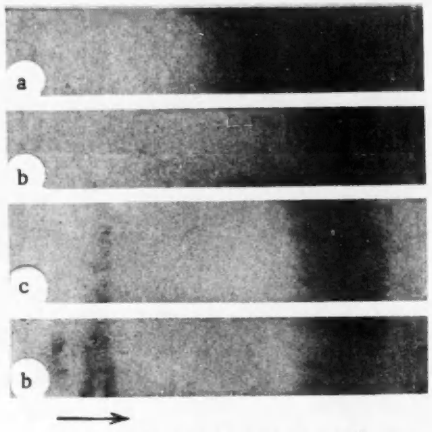


Fig. 2. Electrophoretograms of carp serum proteins before (a and b) and after precipitation by half saturated ammonium sulfate (c and d) stained with bromphenol blue (a and c) and Sudan black (b and d).

TABLE 2. Content (in %) of Liver Proteins Extracted by Salt Solution in Spring Carp

Period after injecting antigen	Fraction			
	IV	*III*	*II*	*I*
	Control			
	16	32	32	21
	15	27	33	25
	14	34	35	17
	3 injections of HS			
7	11	25	38	26
12	12	27	45	26
21	8	35	40	16
27	17	31	40	13
	5 injections of SE			
4	11	29	40	20
10	11	29	39	21

Staining the electrophoretogram of blood serum and supernatant liquid after precipitation by half saturated $(NH_4)_2SO_4$ by the Swahn method shows that in carp (and also in trout and German carp) practically all the lipids of blood serum occur in the fraction which migrates in the electric field along with the α_1-globulin (Fig. 2). The color of the electrophoretogram at the point of deposit of the sample taken after precipitation by $(NH_4)_2SO_4$ and separation from the supernatant liquid by prolonged dialysis against distilled water is evidently produced by partly denatured proteins.

The LP electrophoretogram, as shown in Fig. 2, can be obtained also after precipitation of the protein in the supernatant liquid according to Macheboeuf at pH 3.9 followed by solution in a veronal buffer, pH 8.6.

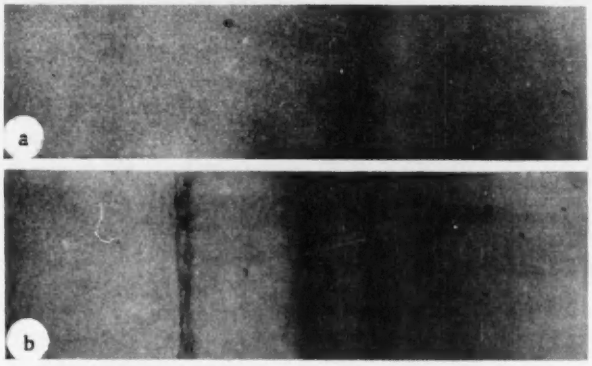


Fig. 3. Electrophoretograms of carp liver proteins extracted with salt solution according to Kaplanskii: a) control; b) after immunization with horse serum. Stain, bromphenol blue.

After multiple injections of HS there occurs in the serum of experimental carp a definite change in the ratio between the different protein fractions (Table 1) and a no less distinct change in the relative content of liver proteins soluble in salt solution (Table 2 and Fig. 3). There is a clear increase in the blood serum of the content of protein which migrates in the electric field with the albumin and α_1-globulin (perhaps due to increased LP content in these fractions), and some decrease in the fraction of β-globulins. The slowest fraction, which could correspond to the γ-globulin fraction of Drihlon, remains unchanged. The fastest fraction, IV in rat livers (Table 2) is immunologically indistinguishable from serum albumin [17]. The clearly

TABLE 3. Direct Determination of the Acid-Alkali Ratio in Blood Serum of Control and Experimental Carp. Figures in parentheses, amount of Cl^- bound in acid medium

Period after injecting antigen, days	Total protein, %	Chloride: mg equiv/liter	Chloride: equiv mole, protein	Wt. titratable substances, mg eq/l	Substances titratable in alkaline medium, mg equiv/liter
Autumn carp		Control			
		75 (25)		36	0
		80 (32)		56	16
		78 (20)		55	23
	3,5	79 (26)	56	54	23
	3,9	109 (95)		45	
	3,1	109 (26)	57	52	21
		10 injections HS			
4		105		44	16
11		95 (29)		45	11
30		103 (24)		43	16
Spring carp		Control			
		100 (90)		53	23
	3,6	71 (27)	50	52	23
	3,3	87 (29)	60	30	0
		3 injections HS			
7	3,6	88 (28)	52	58	26
13	3,4	114 (36)	77	50	34
20	3,2	108 (54)	116	65	23
25	3,2	90 (26)	56	64	34
		5 injections SE			
4	2,9	92 (26)	52	57	19
10	2,9	98 (38)	90	54	22
18	3,0	90 (37)	85	55	20

increased fraction II in carp after injection of a foreign protein is comparable (Table 2) in its mobility with the fraction of β-globulins of blood serum. Immunologically, however, this fraction is not identical with the β-globulin fraction [17].

TABLE 4. Content of Residual and Amino Nitrogen in Serum (S) and Liver (L) of Spring Carp, in mg%

Period after injecting antigen, days	Residual nitrogen		Amino nitrogen		
			total	residual	
	S	L	S	S	L
Control					
	37	171	19,5	4,2	7,2
3 injections HS					
7			26,5	5,8	10,3
13	97,5	409	26,1	10,0	—
20	59,0	—	26,5	11,0	7,4
25	71,8	270	23,5	5,6	8,0
5 injections SE					
4	70,0	315	28,3	5,7	—
10	56,0	429	15,2	7,8	9,5
18	40,5	162	25,8	6,8	

The fact that injection of foreign protein is not indifferent for carp is indicated by the increased content of chloride in the blood serum and especially by the increased ability of serum albumin in the carp treated with foreign protein to bind Cl^- from acid medium. The serum albumin-bound hydrochloric acid, obtained from sodium chloride by elution of the serum from a phenolsulfonate cationite in the hydrogen form, is titrated by the direct method for determining the acid-alkali ratio [18] with a slowly changing conductivity as compared to free HCl. The section of the conductometric curve in which the titration of chloride ends indicates the ability of the serum protein (chiefly the albumin) to bind Cl^-. This value in the control carp, calculated per mole of albumin, is 40-60 equiv, and in the experimental carp it reaches 116 equiv/mole, after injection of HS, and 90 equiv/mole after injection of SE. Damage to some metabolic reaction in the carp which received foreign protein is indicated by a rise in content of residual and amino nitrogen in the blood serum (Table 4) and, in the spring carp, by a rise in the amount of substances titratable in alkaline medium (Table 3).

Uzbekov [19] designates as residual amino nitrogen the amino nitrogen of substances which are not precipitated by protein precipitants. This nitrogen probably corresponds to the basic amino nitrogen of the amino acids and short polypeptides. The amount of residual amino nitrogen in blood serum of experimental carp increases somewhat later, and, in the liver, earlier, and in the latter case more quickly rises to the norm. The dynamics of the content of residual nonprotein nitrogen are analogous. (Table 4).

Evidence for the reaction of carp to the injection of foreign proteins is the change which we observed in content of ATP and in later stages, to a lesser extent, also in lipids (Table 5).

After injection of the foreign protein there was a clear decrease in P_{inorg} content in blood serum and alsc in liver, and also a decrease in ATP content. We observed an increase in DNA content in the liver of experimental carp which could be due only to an increase in content of nuclear elements. Probably there is an accumulation of blood in the liver or an infiltra-

TABLE 5. Content of Phosphorus Compounds in Blood Serum (S) and in Liver (L) (in mg% of fresh tissue) and Activity of Phosphatase in Liver of Control and Experimental Spring Carp

Period after injection of antigen, days	P_{inorg} S	P_{inorg} L	ATP S	ATP L	P-ATP S	P-ATP L	Plipid S	Plipid L	P-RNA S	P-RNA L	P-DNA	P-PP S	P-PP L	Phosphatase activity (in Bodansky units) acid	Phosphatase activity (in Bodansky units) alkaline
Control															
	14,5	45	3		37,5	31,5			4,4		8,8	0,3	1,1	600	74
	10,2	57,5			24,5	23,5	41			50	8,8		1,8		
		39,0			21,0		53			62					
	10,0	31,5	4		20,0	52,0	111							450	46
3 injections of HS															
7	7,4	27,0			13,0	25	65		7,5	52	13	0,4	1,0	407	27
13	8,0	15,5	1,3		17,5	20	63,5		2,4	41	18,8		0,9	440	35
20	6,7	20,2	2,3		9,0	40	116		2,2	63,5	21,5	0,9		480	62
25	7,4	38,0			10,0	38	116			69,5	25,0		2,4	350	50
5 injections of SE															
4	9,0	24,0			14,0	64	132			51,0	23,0		2,8	120	61
10	10,0	37,5			16,0		125			67,0	25,0			650	16
23		42,0			7,0		107			47,5	40,0			620	17

tion of the organ with small cells, and not a change in the parenchyma of the liver itself. Without a parallel histological analysis a final solution of this problem is not possible

As in the rat liver [22] the activity of acid phosphatase in the carp is higher than the activity of the alkaline phosphatase. No change was found in the activity of these in the experimental individuals as compared to the controls. The increased content of PL in the blood serum also reflects the same change in the liver and can be detected by determination of the lipid content by their fatty acids (Table 6).

The results of serological study of blood serum of control and experimental carp in an experiment of long complement fixation are given in Table 7.

TABLE 6. Lipic Content (from Fatty Acids) and Cholesterol in Blood Serum of Control and Experimental Spring Carp

Period after injection of antigen, days	Fatty acids of lipids, mg equiv /liter			Total cholesterol, mg%
	total amount	acetone-soluble	acetone-insoluble	
Control				
	35,0	19,0	8,0	183
	29,5		16,0	
	22,0		17,0	152
	26,6	12,0	24,8	
3 injections of HS				
7	32,0	15,0	14,0	191
13	28,0			188
20	58,0	23,0	35,0	182
25	43,0	5,0	44,0	
5 injections of SE				
4	35,0	8,0	22,0	224
10	23,0		20,0	160
18	32,0	10,5	24,5	186

TABLE 7. Results of Study of Carp Serum in Experiments of Long Complement Fixation at 0°

Number of HS injections and period of taking blood, in days	Dilution of fish serum	Normal HS 1:100 and complement in dose			Control (fish serum with addition of complement), in dose		General estimation of reaction
		1	1.5	2	1	1.5	
Autumn carp							
6, 8	1:10	−	−	−	−	−	−
	1:50	−	−	−	−	−	−
	1:100	−	−	−	−	−	−
10, 4	1:10	−	−	−	−	−	−
	1:50	−	−	−	−	−	−
	1:100	−	−	−	−	−	−
10, 8	1:10	++	+	−	−	−	−
	1:20	−	−	−	−	−	−
	1:50	−	−	−	−	−	−
10, 25	1:10	−	−	−	−	−	−
	1:20	−	−	−	−	−	−
	1:50	−	−	−	−	−	−
Spring carp							
3, 7	1:10	+++	+	−	++	−	±
	1:40	++	−	−	−	−	−
	1:160	++	−	−	−	−	−
3, 23	1:80	++	+	−	+	−	±
	1:160	++	+	−	−	−	±
	1:320	+	−	−	−	−	−
3, 26	1:160	++++	++++		++++		±
	1:320	++++	++				+
	1:640	+	−	−	−	−	−
Control serum of intact carp	1:10	−	−	−	−	−	−
	1:40	−	−	−	−	−	−
	1:160	−	−	−	−	−	−
Control (normal HS + +complement)		−	−				

Each of the dilute samples of fish serum was studied at three different complement concentrations (1, 1.5, and 2 doses of complement). On the basis of these three parallel studies we evaluated the reaction . According to the generally accepted system, the maximum reaction intensity, which in this case corresponds to a high antibody content, is denoted by four plus (complete prevention of hemolysis ++++), a doubtful result is indicated by ±, a negative one by a minus sign-. Intermediate degrees of intensity of serological reaction are indicated by one, two, or three plus signs.

Binding of the complement against horse serum was not observed in even one of the seven samples of carp blood taken at different periods after immunization. We can assume that the prevention of hemolysis observed in some of the tubes in the experiments with spring carp depended not on the presence of fixation of the complement by "anti-horse serum" antibodies, but on nonspecific adsorption of the complement. This is also indicated by the prevention of hemolysis in a number of control tubes which do not contain horse serum antigen.

These samples of serum from the spring carp were studied in precipitation experiments . A precipitation ring appeared in experiments with all samples of serum of immunized carp. However, this phenomenon was also nonspecific. The serum of control carp produced precipitation of horse serum proteins. This fact again confirms the previous suggestion of the unsuitability of phenomena of precipitation, agglutination, and cytolysis to show immunological reactions in lower vertebrates and invertebrates [23], since in the blood and hemolymph of the latter there are very often substances which cause precipitation of soluble proteins, agglutination of bacteria, or solution of foreign cells.

The same negative data were also obtained in experiments on immunization of carp by SE. The serum of carp taken at different periods after repeated injection of foreign erthrocytes did not show hemolysis.

SUMMARY

Parenteral injection into the organism of carp of horse serum and sheep erythrocytes did not cause formation of specific antibodies and with unchanged total protein content in the blood serum did not lead to increase in the fraction of proteins which corresponds in mobility to the γ-globulin of other animals.

After injection of foreign protein in the carp serum there is a relative increase in content of fractions which migrate in the electric field with the albumin and α_1--globulin fractions (in all probability due to increased content of their lipoproteins), and a rise in the content of fraction II (of Kaplanskii) in liver proteins. Serum albumin in the experimental carp gains an increased ability to bind Cl^- from acid solution.

In the blood serum and liver of experimental carp there is an increase in content of residual nitrogen, total amino and residual amino nitrogen, and a decrease in ATP content. There is also an increase of lipids insoluble in acetone. The DNA content in the liver increases.

LITERATURE CITED

[1] Ya.A. Épshtein and N.F. Lavrovskaya, Biokhimiya 24, 592 (1959).†

[2] V.G. Avetikyan, Proceedings of the Conference on Physiology of Fish [in Russian] (Izd. AN SSSR, Moscow, 1957), p. 387.

[3] A.C. Drihlon, Compt. rend. Acad. Sci. 237, 1779 (1953); 238, 946 (1954).

[4] R.L. Engle, K. Woods, E.C. Paulsen, and J.H. Pert, Proc. Soc. Exptl Biol. and Med. 98, 905 (1958).

[5] V.F. Deutsch and M.H. McSchan, J. Biol. Chem. 180, 219 (1948).

[6] K.F. Sorvachev, Biokhimiya 22, 872 (1957).†

[7] H. Flemming, Z. Fischerei 7, 91 (1958).

[8] K.A. Bisset, J. Hyg. 45, 128 (1947).

[9] J.E. Cushing, J. Immunol. 45, 123 (1942).

[10] J.E. Cushing, and D.H. Campbell, Principles of Immunology (Mc Graw-Hill, New York, 1957).

[11] B. Swahn, Scand. J. Clin and Lab. Invest. 5, suppl. 9 (1953).

[12] M. Macheboeuf, Bull. Soc. chim. biol. 11, 485 (1929); Disc. Faraday Soc. 6, 62, (1949).

[13] N.P. Lisovskaya, Biokhimiya 19, 626 (1954).

[14] D.N. Yakhina, Biokhimiya 21, 429 (1956).†

[15] Ya.A. Épshtein, Biokhimiya 5, 162 (1940).

[16] K. Schmidt-Nielsen, Compt. rend. Lab. Carlsberg. Ser. chim. 24, 233 (1942).

[17] S.Ya. Kaplanskii and A.E. Gurvich, Biokhimiya 23, 114 (1958).†

[18] Ya.A. Épshtein, Biokhimiya 16, 572 (1951).

[19] G.A. Uzbekov, Voprosy Med. Khim. 4, 69 (1958).

[20] S.M. Neuschloss, Biochem. Z. 225, 11 (1930).

[21] S.D. Balakhovskii and I.S. Balakhovskii Methods of Chemical Analysis of Blood [in Russian] (Medgiz, Moscow, 1953), p. 626.

[22] Z.S. Tepikina, Tr. Stalinabadsk. Med. Inst. 27, 59 (1957).

[23] B.G. Avetikyan, in: Experimental and Clinical Immunology [in Russian], (Inst. Exper. Med., Leningrad, 1959).

† Original Russian pagination. See C.B. Translation.

ON THE PROPERTIES AND INTRACELLULAR DISTRIBUTION OF THE L- AND D-AMINO ACID OXIDASES IN THE KIDNEY

A.V. Azyavchik

Physiological Chemistry Laboratory, Institute for Biological and Medical Chemistry, Academy of Meical Sciences USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 436-442, May-June, 1960

Original article submitted August 12, 1959

Claude [1] demonstrated as early as 1946 that D-amino acid oxidase in liver cells was localized mainly in the mitochondria. Khesin [2] confirmed this observation and showed that the specific activity of the mitochondrial fraction was 4-5 times that of the whole homogenate. No published data, however, could be found on the localization of D-amino acid oxidases in the cells of other tissues, particularly in the kidney, where very active D-amino acid oxidases are known to be present. We have also been unable to find any published data on the localization of L-amino acid oxidases in either liver or kidney cells. In view of the fact that a detailed survey of the intracellular localization of enzymes, concerned with amino acid metabolism, is presently being conducted in this laboratory [3], we thought it desirable to attempt to define the intracellular distribution of these enxymes in the kidney, and to isolate such enzymes in a purified state from some of the intracellular elements in similarity to the previously described [4] isolation from liver of the enzymes catalyzing amino acid synthesis from keto acids and ammonium salts.

METHODS

White rats, weighting 120-180 g, were used. The kidneys were excised immediately after killing the animals and were homogenized in all all-glass homogenizer with cold 0.44 M sucrose in KCl-phosphate buffer. The suspensions were centrifuged in the cold at 800 rpm in order to bring down nuclei and debris, while mitochondria were collected by centrifuging at 12,000 rpm. The fractionation thus yielded three fractions: the nuclear, mitochondrial, and supernatant, the latter containing microsomes and the hyaloplasm. The nuclear fraction was further purified according to Schneider et al. [5]. Each fraction was assayed for L- and D-amino acid oxidase activity, using pure L- and DL- alanine as a substrate (final concentration, 0.05 M). The use of DL-alanine as a substrate for D-amino acid oxidase was necessitated by the nonavailability of pure D-alanine in sufficient amounts. It was established in control comparative experiments that the activity with D-alanine was higher (400-600 μ moles/g tissue) than with DL-alanine (280-400 μ moles/g tissue). However, since the L-amino acid oxidase activity did not exceed 30 μ moles of N-NH_3 per g tissue, it was clear that, when DL-alanine was used as a substrate, the main activity assayed was D-amino acid oxidase. The DL-amino acid oxidase activity was assayed as ammonia produced after a 2-hour incubation at 38° and in the presence of 1/400 M arsenite, which was added in order to prevent secondary synthesis (amination of keto acids and glutamine synthesis). Incubation mixtures, complete with the exception of substrate, were used as controls. Ammonia was estimated according to Conway. The isolation of L- and D-amino acids from kidney mitochondria was carried out after disruption by repeated thawing and freezing in a mixture of ice and salt.

RESULTS

The results dealing with the intracellular distribution of the L- and D-amino acid oxidases in the kidney are shown in Table 1.

It will be seen from the table that the activity D-amino acid oxidase is concentrated mainly in the mitochondria, where its activity was about twice that of the crude homogenate, and four to five times that of any one of the subcellular fractions. On the other hand, the L-amino acid oxidase was found to be uniformly distributed between all the subcellular fractions, its activity in the supernate being about the same as that in the mitochondrial fraction.

The relative distribution of the two oxidases in various fractions is noteworthy. Inthe crude homogenate and in the mitochondrial fraction the relative ratio of D- to L-amino acid oxidase activity was 10:1; in the nuclear fraction it fell to 7:1, while in the supernate it was 3:1. Disruption of mitochondria had a highly destructive effect on D-amino acid oxidase activity, which fell from 26 μ moles NH_3-N /mg protein-N to 4.01 μ moles—less than one sixth.

On the other hand, L-amino acid oxidase suffered no inactivation during mitochondrial disruption; in most cases definite increase in activity was observed, the mean change being from 2.12 μ moles NH_3-N/mg protein -N to 3.82 μ moles. This increase in activity, coupled with the sharp decline in D-amino acid oxidase activity in the disrupted mitochondria, resulted in equal levels of activity of the two enzymes in this fraction. In order to elucidate the possible causes for this diverse response to freezing and

TABLE 1. Deamination of D- and L-Alanine by Various Subcellular Fractions of the Kidney. (In μ moles NH_3-N /mg protein-N; 2-hour incubation at 38°)

Homogenate		Nuclei		Supernate		Intact mitochondria		Disrupted mitochondria	
L-alanine	DL-alanine	L-alanine	DL-alanine	L-alanine	DL-alanine	L-alanine	DL-alanine	L-alanine	DL-alanine
0,80	12,01	1,62	10,68	0,65	3,70	1,60	22,50	1,20	4,00
0,95	15,00	1,48	9,18	2,30	7,50	3,20	26,00	2,51	6,01
1,27	12,60	1,22	9,70	2,75	5,26	1,60	13,00	5,20	5,30
2,06	9,18	1,52	10,18	1,85	4,80	1,45	29,00	5,70	6,80
1,20	16,80	1,01	11,01	1,23	5,11	2,47	19,00	3,89	2,70
1,82	17,10	1,29	9,60	1,94	4,98	1,98	35,90	2,50	2,22
1,72	9,06	1,42	7,70	1,80	5,29	2,00	25,17	1,66	3,20
1,31	17,17	—	—	0,80	6,50	1,60	32,01	1,91	3,61
1,72	13,00	—	—	2,69	6,40	2,00	26,60	1,66	2,72
1,82	16,31	—	—	1,60	4,98	3,21	17,62	3,70	2,60
—	18,70	—	—	0,90	5,11	2,42	32,07	9,06	4,21
—	18,90	—	—	2,10	6,47	1,98	26,88	9,04	2,60
—	12,42	—	—	1,80	—	—	31,84	1,62	2,70
—	18,20	—	—	—	—	—	—	—	5,30
—	12,60	—	—	—	—	—	—	—	6,00
—	12,62	—	—	—	—	—	—	—	4,31
—	12,43	—	—	—	—	—	—	—	—

thawing of the two enzymes both activities were assayed again not only in the supernate after centrifuging the frozen-thawed mitochondria, but also in the pellet. The following results(in μ moles NH_3-N /mg protein-N) were obtained:

L-amino acid oxidase	D-amino acid oxidase
0.91	6.70
1.46	9.11
0.81	9.56
1.10	8.30
1.08	7.95

As can be seen from the results, a considerable portion of both oxidase activities came down with the precipitate after centrifugation of the disrupted mitochondria. This could naturally be expected to lower the total activity of the mitochondrial fraction. However, the specific activities of both enzymes in the pellet protein were lower than those of the intact mitochondria. This circumstance suggested that the specific activities in the supernate should be higher than those of the intact mitochondria and, indeed, such was the case with the L-amino acid oxidase. But the D-amino acid oxidase specific activity in the supernatant was lower than that of the intact mitochondria, and this indicated the necessity for another approach. It may have been argued that the disruption of mitochondria may cause the removal from the enzyme, or the destruction of the coenzyme of D-amino acid oxidase; FAD. Should that be the case, the activity of the disrupted mitochondria should be restorable simply by the addition of a boiled liver or kidney extract, containing FAD. Such re-activation is always demonstrated with the purified D-amino acid oxidase from which the FAD has been split. However, the addition of a boiled kidney extract, alone or with DNP, failed to restore the activity of disrupted mitochondria. The remaining explanation is that the repeated freezing and thawing of the mitochondria caused some changes in the enzyme protein itself which brought about a partial loss of activity. The latter assumption is now being checked experimentally. It appears that freezing and thawing of mitochondria has no effect on the protein of the L-amino acid oxidase complex.

Attempts were now made to purify the L- and D-amino acid oxidases from disrupted mitochondria, using starch-block electrophoresis.

Mitochondria isolated from 15 g of kidney tissues were suspended in 10-15 ml of sodium phosphate buffer at pH 7.4 and subjected to repeated freezing and thawing. The suspension was centrifuged for 15 min at 18,000 rpm and the supernate (8-10 ml) was transferred to the starch block. Electrophoresis was carried out for 18-20 hr, with a current of 25-27 mA and potential difference of 400 v , in phosphate buffer, pH 7.4. At the completion of the run the starch block (26 cm) was sectioned into either 13 or eight sections, which were extracted individually with Ringer phosphate. All extracts were then assayed for protein according to Lowry [6] and for L- and D-amino acid oxidase activity. The results are shown in Tables 2 and 3.

It will be seen from Table 2 that the end of the electrophoretic run D-amino acid oxidase activity was distributed throughout the starch block. However, the terminal fractions near the anode usually contained more activity than any other fractions. Thus, the two terminal sections nearest to the anode (fractions 1, 2) contained protein with a specific activity of 20 μ moles NH_3-N /mg, which was 10-30 times that of the initial extract.

The distribution of the L-amino acid oxidase at the end of the electrophoretic run resembled that of the D-

TABLE 2. The Activity of D-Amino Acid Oxidase in Various Fractions Obtained by Starch Block Electrophoresis.
(In μ moles NH_3 -N Split from DL-alanine per 1 mg protein. 2-hour incubation at 38°)

Initial activity in mito. extract	Fraction Number												
	anode	2	3	4	5	6	7	8	9	10	11	12	cathode
Block divided into 13 fractions													
1,88	0,00	50,00	213,40	120,00	37,10	21,40*	—	—	34,30	22,10	20,90	28,50	151,00
1,77	—	120,00	50,00	19,2	—	16,10*	14,00	16,30	88,70	58,10	43,10	194,00	87,20
2,98	—	107,00	50,61	—	—	87,60*	22,81	37,60	34,60	28,10	61,40	74,30	32,40
1,95	0,00	74,90	34,30	14,50	—	87,50*	10,50	34,37	30,43	61,50	—	—	—
0,66	0,00	18,00	118,00	43,22	13,00	25,41*	32,20	42,60	33,10	10,15	13,26	8,10	196,70
2,70	50,00	140,00	200,40	200,10	98,10	172,40*	17,40	19,01	22,50	13,70	12,50	130,60	80,47
Block divided into eight fractions													
2,30	137,00	176,00	50,00	—*	82,50	—	92,10	28,00					
3,00	122,50	276,00	120,00	11,40*	41,80	85,70	43,70	110,00					
1,80	71,10	43,00	91,40	19,40*	28,10	11,90	80,00	49,20					
2,8	159,00	—	143,20	—*	46,90	81,80	128,00	146,80					

*Origin.

TABLE 3. The Activity of L-Amino Acid Oxidases In Individual Fractions Obtained By Starch Block Electrophoresis
(In μ moles NH_3 -N split from L-alanine per 1 mg protein. 2-hour incubation, 38°)

Initial activity disrupted mito. extract	Fraction number												
	anode	2	3	4	5	6	7	8	9	10	11	12	cathode
Block cut into 13 fractions													
1,77	—	107,00	50,00	—	18,00	22,80*	37,00	34,60	—	28,10	32,00	30,00	—
0,60	18,10	36,89	30,79	21,71	28,56	8,28*	—	34,30	12,07	11,80	18,44	18,00	6,00
5,20	50,00	181,10	62,50	12,50	50,00	23,00*	5,00	25,80	42,60	19,40	3,10	13,90	—
3,20	52,70	130,00	53,30	19,40	30,41	104,00*	36,50	13,91	13,70	4,83	12,20	6,70	4,20
0,98	0,00	9,00	96,10	21,60	—	25,40*	21,30	—	5,20	7,60	—	40,50	5,46
4,80	25,20	45,00	18,01	81,03	—	15,00*	—	12,00	—	30,50	15,20	34,20	—
1,10	20,00	54,90	20,00	10,00	—	6,60*	10,40	34,30	15,20	32,00	10,00	11,00	0,00
Block cut into eight fractions													
4,80	35,70	39,80	102,80	12,00*	114,70	17,20	80,00	36,40					
5,20	136,29	40,00	15,90	3,47*	10,00	13,33	10,97	48,00					
4,20	130,00	22,20	28,00	—*	43,90	—	32,30	28,00					

*Origin

amino acid oxidase inasmuch as the activity could be detected throughout the block, with the highest peak being concentrated in the vicinity of the anode (fractions 2, 3, 4). However, a distinct feature here was the appearance in some cases of a strong L-amino acid oxidase activity peak in the terminal sections near the cathode (fractions 12 and 13). This was more noticeable when the block was sectioned into eight sections instead of the usual 13 2-cm sections. In the majority of cases the activity in the first terminal section adjacent to the anode and in the two terminal sections adjacent to the cathode exceeded the value of 100 μ moles NH3-N per mg protein, while the central sections contained protein with a mean activity value of 20-30 μ moles NH_3-N /mg. The results with both D- and L-oxidase indicate that starch block electrophoresis could not provide a satisfactory method for the separation of the two enzymes into two separate peaks. The dual distribution of L-amino acid oxidase activity in the two terminal portions of the starch block suggests the presence of two enzymes, of which one travelled towards the anode, while the other either travels to the cathode by virtue of its charge, or (which is more likely) has almost no net charge and is transported by electroosmosis. These observations are in agreement with those of Green et al. [7], who succeeded in the separation of L-amino acid oxidase on the ultracentrifuge into two fractions: one light and the other four times heavier. According to Green, the heavy component consists of aggregates of four single L-amino acid oxidase molecules, the latter being present in the lighter component.

It was of interest from the point of view of further study to prepare some of the active fractions, obtained by electrophoresis, in the dry state, in which they could be stored for considerable time. Both L- and D-amino acid oxidase preparations eluted from the starch block sections containing highest activity were dialyzed and lyophilized. Further checks of the lyophilized preparations showed that neither of the two enzymes lost any activity when storedin this state at 4° for at least six months.

SUMMARY

Kidney L-amino acid oxidase differs from the D-amino acid oxidase in that it is equally distributed among all the subcellular fractions of the kidney cell: nuclei, mitochondria, and supernatant.

The specific activity of L-amino acid oxidase in an extract of disrupted mitochondria was found to be higher than in a similar extract of intact mitochondria. D-amino acid oxidase activity fell sharply when mitochondria were disrupted by repeated freezing and thawing. This loss of activity was shown to be due to causes other than the splitting or the destruction of the coenzyme. Electrophoresis on a starch block of the extract of disrupted mitochondria resulted in a distribution of D-amino acid oxidase background activity throughout the block, but the peak of activity was concentrated in the terminal sections adjacent to the anode. Under the same conditions L-amino acid oxidase was found to distribute itself into two main peaks, one migrating towards the anode, the other towards the cathode. This observation suggests that L-amino acid oxidase consists of two components, a conclusion in support of that reported by Green and coworkers.

Both L- and D- amino acid oxidase can by lyophilized without loss of activity and lyophilized preparations of both enzymes have now been stored at 4° and remained active for at least six months.

I wish to express my thanks to Prof. S.Ya. Kaplanskii for the supervision of the work reported above.

LITERATURE CITED

[1] A.A. Claude, Sci. Res. Conf. on Cancer (1945). p. 223

[2] R.V. Khesin, Doklady Akad. Nauk SSSR 23, 359 (1950).

[3] N.N. Berezovskaya, Biokhimiya 21, 733 (1956).*

[4] N.N. Berezovskaya, Biokhimiya 23, 125 (1958).*

[5] G.H. Hogeloom, W.C. Schneider and M.J. Striebich, J. Biol. Chem. 196, 111 (1952).

[6] O.H. Lowry, W.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem. 193, 265 (1951).

[7] M. Blanchard, D.E. Green, V. Nocito, and S. Ratner, J. Biol. Chem. 161, 2, 583 (1945).

*Original Russian pagination. See C.B. Translation.

THE BINDING OF LABELED AMINO ACIDS BY PROTEIN AND NUCLEIC ACID PREPARATIONS

O.P. Samarina, I.B. Zbarskii and K.A. Perevoshchikova

A. N. Severtsov Institute of Animal Morphology, Academy of Sciences USSR, and the P. A. Gertsen State Oncological Institute, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 443-451, May-June, 1960
Original article submitted August 19, 1959

The incorporation of labeled amino acids is at present one of the most widely used criteria for protein synthesis in the study of this process both in vivo and in vitro, including studies in cell-free systems. However, incorporation of radioactivity can, apparently, be due not only to actual protein neosynthesis, but is also a result of an exchange reaction, that is, a substitution of amino acid residues in the protein molecule [1, 2].

It is also known [3, 4] that in simple systems, proteins are capable of binding radioactive amino acids. This phenomenon must be taken into consideration particularly carefully when cell-free preparations are being used, since there, particularly, any conclusions about true protein synthesis must be treated with utmost caution [5]. In fact, in the study of labeled amino acid incorporation into isolated cell nuclei [6-8], the blank values are extremely high and, in some cases approach those of the actual experiment.

The mechanism of amino acid interaction with proteins is still not clear and is subject to considerable controversy [9-14].

In contrast to opinions that the process is a purely physical adsorption phenomenon, there are also theories that it reflects true protein synthesis, inherent in isolated protein molecules [14]. Although the latter theory is extremely doubtful, particularly as the phenomenon proceeds more actively at high temperatures (100-120°), it is quite possible that it is still closely related to the initial stages of protein biosynthesis.

Investigations in this direction have revealed that the process of amino acid binding by proteins is intensified at temperatures above denaturation point, and that it depends on the nature of the protein and of the amino acids which participate, in the reaction. Furthermore from the published data the process does not appear to show a proportional correlation with time.

These observations indicate that the process cannot be a true enzymatic one, and that it is more likely to be a physico-chemical interaction between proteins and amino acids.

It has occurred to us that the different intensities with which amino acids are bound by different protein preparations may be due to the presence of nonprotein groupings, particularly nucleic acids, within the protein preparations. The suggestion was in keeping with the known important role of nucleic acids and nucleoproteins in protein synthesis. Furthermore, in previous experiments, the binding of amino acids by protein preparations was not correlated with the presence in these preparations of nucleic acids which, apparently, can also bind amino acids [20, 21].

The present communication deals with the binding of labeled amino acids glycine, tyrosine, lysine and methionine, by various preparations of proteins and nucleoproteins, as well as by nucleic acids and by other biological polymers (starch and glycogen).

METHODS

The protein preparations used were: unfractionated proteins of rat serum and egg white, histone obtained by extraction with 0.5 M HCl, of DNP * from calf thymus and crystalline chymotrypsin prepared from beef pancreas. In some experiments commercial preparations of protamine sulfate (from salmon sperm) and beef pancreatic ribonuclease were also used. DNP was extracted from calf thymus with 1 M NaCl after washing the minced tissue with 0.14 M NaCl; it was also prepared from cell nuclei isolated from rat liver, calf thymus or from rat sarcoma M_1.

Rat liver RNP and rabbit pancreas RNP were isolated from aqueous extracts of the minced tissue at pH 8.0. The extracts were acidified to pH 6.0 with acetic acid, the precipitate formed was rejected and the supernatant was adjusted to pH 4.0 with acetic acid; the resulting precipitate was collected and used. In some cases the globulin fraction and the acid protein of isolated rat liver nuclei [15] was used as RNP preparation.

The nucleic acid used was obtained commercially from a variety of sources: sodium ribonucleate from the L.Ya. Karpov factory, free RNA from "Chinoin" (Hungary) and from "La Chema" (Czechoslovakia); high RNA and DNA polymers were also prepared from rat liver, and DNA from calf thymus, by a method developed in this laboratory [16]. The starch used was a domestic

* The following abbreviations will be used: DNA—desoxyribinucleic acid; RNA—ribonucleic acid; DNP—desoxyribonucleoprotein; RNP—ribonucleoprotein; TCA—trichloroacetic acid.

commercial preparation and the glycogen was prepared from rabbit liver [17].

Reaction mixtures were incubated for 30 min at 38°, usually in phosphate buffer pH 7.4, in Warburg apparatus or in the thermostat. Reaction mixtures of a final volume of two ml contained usually about 10 mg of the preparation investigated and the labeled amino acid with a total activity of 200,000–300,000 cpm. The experimental conditions were varied on a number of occasions, but it was invariably found that saturation with oxygen, aeration, or composition of the medium (distilled water, phosphate buffer or Robinson medium) had no effect on the final result. It was also found, as a result of time–activity studies, that the incubation time bore no direct relation to the degree of amino acid incorporation.

At the end of each incubation the reaction mixtures were precipitated by the addition of 50% TCA to a final concentration of 5-7% and two volumes of ethanol or acetone. The precipitated protein, nucleoprotein or nucleic acids were washed with 5% TCA, then 7-9 times with ethanol or with acetone, and air-dried. Radioactivity was estimated by counting the solid in a thin layer with an end-window counter Type B [10, 18]. The results were expressed as relative specific activity (RSA), either as cpm/g of the preparation relative to total cpm/reaction mixture, or as per cent bound radioactivity (amino acid) relative to that present in the reaction mixture.

Where the stability of the bonds formed was investigated, the preparations were redissolved in alkali (0.01 N NaOH), or were treated with ninhydrin at pH 2.3 [7], reprecipitated and, after two washes, were dried with acetone and ether; radioactivity in samples thus treated was again estimated as above.

RESULTS

As mentioned above, when various preparations of proteins or nucleoproteins were incubated with labeled amino acids, the incorporation of label did not proceed linearly with time. In the majority of cases, but by no means in all cases, prolonged incubation resulted in a somewhat increased degree of incorporation, but no fast rule for such relation could be made. Incorporation was also considerable in the nonincubated controls, where values were not greatly different from those observed in the actual reaction mixtures. It is important to note that the absolute values for amino acid binding were subject to considerable scatter, but on all occassions nucleoproteins bound more label than proteins, while with nucleic acids the incorporation was the highest of all preparations. Starch and glycogen also bound a certain amount of amino acids, but the bond formed was less stable than in the case of the other compounds.

The effect of repeated washing with 5% TCA and with acid ethanol on the binding of labeled tyrosine by RNA, starch and glycogen is shown in Table 1.

Although the individual results were subject to considerable variation, it is obvious that, while starch and glycogen gradually lost their label with washing, the RNA retained the label present after the first wash.

Presented below are the results of the binding of various labeled amino acids by some preparations of proteins, nucleoproteins and nucleic acids (the values are compared with that of serum protein, which was taken as unity):

These results (values shown are means of 8-10 determinations) show that RNP and DNP retained and bound considerably higher amounts of labeled glycine, tyrosine and lysine than did the pure proteins which contained no nucleic acid goupings. Furthermore, the degree of binding increased with the nucleic acid content of the preparation. The binding of glycine and lysine by the two nucleic acid preparations, and the binding of tyrosine by DNA was 30-40 times more intensive than binding of these amino acids by proteins, and only in the case of tyrosine binding by RNA was the factor somewhat lower–9.6. This more intensive uptake of tyrosine by DNA compared with its reaction with RNA was the only difference observed between RNA and DNA in amino acid binding.

A comparison of the various polymers within each individual group showed that with the exception of the serum proteins, the members of the groups, such as the proteins containing no nucleic acid residues, the nucleoproteins and the nucleic acids, showed comparable capacities for amino acid binding.

Presented in Table 2 is a comparison of the binding capacity for labeled amino acids of native nuclear nucleoproteins with that of artificially produced nucleoproteins obtained by complexing RNA or DNA with several proteins of a basic nature. There is no established

TABLE 1. The Binding of C^{14}-Tyrosine and S^{35}-Methionine by Ribonucleic Acid, Starch, and Glycogen. All values as RSA: cpm/g preparation per cpm/2 ml of incubation mixture

Preparation	C^{14}-tyrosine							S^{35}-methionine		
	unwashed	six washes, 5% TCA	washed in 70% ethanol pH 4.5 — 5 times	10 times			7 times	unwashed	washed — 5% TCA, 6 times	70% EtOH pH 4.5, 10 times
RNA	4,9	2,30	1,9	3,1	2,4	1,2	2,4	8,0	4,4	5,1
Starch	0,6	0,26	1,1	0,25	0,07	0,06	0,08	2,4	1,3	0,8
Glycogen	—	0,27	0,38	0,18	—	—	—	—	1,3	—

Preparation	C^{14}-glycine	C^{14}-tyrosine	C^{14}-lysine
Serum protein	1	1	1
Rat liver RNP	2,6	2,7	3,2
Pancreas RNP	4,0	5,2	5,0
Liver and thymus DNP	6,2	6,2	8,5
RNA	37,0	9,6	33,1
DNA	34,2	45,3	35,0

case for the binding of glycine and lysine by complexes of DNA with ribonuclease or chymotrypsin or by RNA complexes with protamine or with chymotrypsinogen, since the number of experiments carried out (from 1 to 4) was too low; it is, however, noteworthy that the high values for binding observed in these cases corresponded with a complex between a nucleic acid and proteins with which the nucleic acid is not usually combined, and where some proportion of the active groups of the nucleic acid could be expected to remain free. The binding of amino acids by DNA in combination with protamine was essentially the same as by native DNP.

TABLE 2. The Incorporation of Labeled Amino Acids by Native and Artificial Nucleoproteins. Mean values, RSA = (cpm/g preparation):(cpm per test [2 ml])

Preparation	C^{14}-glycine	C^{14}-tyrosine	C^{14}-lysine
Nuclear DNP	0.23	0.41	0.82
DNA-protamine	0.25	0.20	0.20
DNA-chymotrypsinogen	–	0.41	2.7
DNA-histone	–	0.11	
DNA-ribonuclease	1.7	0.64	2.1
Rat liver nuclear RNP	0.12	0.35	
RNA-protamine	1.6	0.64	2.0
RNA-chymotrypsin	–	0.18	3.2

The stability of the bonds formed during the incorporation of amino acids is compared in Table 3.

Proteins have been shown to retain the major portion of radioactivity when treated with alkali or with ninhydrin (the two procedures produced the same result). Similar treatment of the nucleic acids resulted in the loss of the bulk of radioactivity. In the nucleoproteins the bulk of the radioactivity, comparable in intensity to that retained by the proteins, was also retained throughout the treatment. The results dealing with the extraction with hot TCA, which served to remove nucleic acids, indicate that the high radioactivity was associated with the protein moiety of the necleoproteins.

It is of interest to note that the artificially combined nucleoproteins, of a composition alien to that of the native nucleoprotein, retained a relatively lower proportion of the label present before treatment. It should, however, be remembered that, since these complexes usually bound amino acids more intensively (Table 2), the absolute amount of radioactivity retained by them after treatment was in some cases the same as in the native material. In the case of nucleic acids the radioactivity retained after treatment, although representing

TABLE 3. Retention of Radioactivity After Binding of Labeled Amino Acids by Proteins, Nucleoproteins and Nucleic Acids. (percent radioactivity remaining after treatment)

Preparation	C^{14}-glycine		C^{14}-tyrosine		C^{14}-lysine
	alkali or ninhydrin	hot TCA	alkali or ninhydrin	hot TCA	alkali or ninhydrin
Protein	93		77		58
Rat liver nuclear DNP	75	81	52	82	
Rat sarcoma M_1 nuclear DNP			96	60	
Calf thymus nuclear DNP	34		20	69	51
DNA-protamine	8		27	80	36
DNA-chymotrypsinogen	4		14	30	9
DNA-ribonuclease					9
Rat liver nuclear RNP	62		50		
RNA-protamine			18		8
DNA	14		11		15
RNA	3		5		6

only a small proportion of that originally held, was still higher in absolute amounts than that retained by proteins.

The binding of amino acids by nucleic acids, and the effect of varying experimental conditions on this phenomenon, was further investigated.

The variation of the composition of incubation medium showed very little effect on the binding of tyrosine and glycine by RNA (% total radioactivity bound), and only in strongly acid solutions was there any noticeable decrease in the degree of binding:

Incubation medium	C^{14}-glycine		C^{14}-tyrosine	
	expt 1	expt 2	expt 1	expt 2
Double-dist. H_2O	3,7	—	1,2	—
Phosphate buffer, pH 7.4	3,0	2,3	1,0	1,5
	2,2		0,5	
Robinson's medium	—	1,3	—	1,0
	—	0,4	—	0,3

Nevertheless, the considerable binding activity of RNA in 0.1 N HCl, and particularly in 1 N HCl, indicates that the binding of amino acids by RNA can take place over a wide range of salt concentration and pH. The presence of salts (cations and anions) did not catalyze the reaction, but rather inhibited the binding slightly. The binding of glycine and of tyrosine in 1 N HCl was about one-fourth to one-fifth of the value in phosphate buffer.

Attempts to demonstrate some time sequence of the binding reaction with labeled glycine showed that the value for binding was at maximum already at 15 min and failed to increase further in the subsequent incubation time up to two hours. The initial value (zero time), obtained with a mixture which was precipitated with TCA immediately after mixing the components, was also the same as at 15 min, thus showing that the binding process was very rapid.

The binding of labeled glycine by RNA preparations of various origin was also tested. With two of the Na salts of RNA available, and with one sample of free RNA, the values obtained for glycine binding were virtually the same, notwithstanding the difference in solubilities of these preparations in the incubation medium. Only one preparation (of Hungarian manufacture) showed a somewhat lower binding ability, possibly because of a lower degree of purity.

Determinations of the comparative degree of binding by RNA of C^{14}-labeled glycine and tyrosine, and of S^{35}-methionine (in per cent of total radioactivity bound) showed the following:

Amino acid	Expt. No. 1	Expt. No. 2
C^{14}-glycine	3,7	3,6
S^{35}-methionine	2,3	1,5
C^{14}-tyrosine	1,2	1,0

Glycine is thus bound most intensively by RNA, while methionine and tyrosine were bound in a decreasing order of intensity.

The following results were obtained by comparing the relative degree of binding of labeled glycine and tyrosine by different preparations of RNA and DNA (in per cent total radio activity).

Polymer	Amino acid	Expt. 1	Expt. 2	Expt. 3*	Expt. 4*
RNA	C^{14}-Tyrosine	0,85	0,5	1,6	1,4
RNA	C^{14}-Glycine	2,4	1,7	4,8	3,7
DNA	C^{14}-Tyrosine	3,0	2,1	1,5	1,0
DNA	C^{14}-Glycine	2,5	1,0	1,2	1,2

* Highly polymerized preparations of RNA and DNA were used.

The DNA preparation used ("Chinoin", 70% pure) bound tyrosine somewhat more actively than it did glycine. Tyrosine binding by DNA was invariably higher than by RNA. The reverse held for glycine. It will also be noted that the highly-polymerized RNA and DNA preparations bound amino acids to approximately the same extent as the commercial preparations.

The ability of RNA and DNA to bind amino acids was thus established. This binding was found to be scarcely affected by the pH of the reaction medium, by the presence of salts or by their absence (cf. reaction in doubly distilled water), and could proceed in highly acid medium (1 N HCl). The amino acids investigated– glycine, methionine and tyrosine– also differed from each other in the degree of their binding by RNA. Although the degree of binding of any one amino acid varied to some extent from experiment to experiment, the actual ratio of the degree of binding of the amino was invariably the same. The binding of the various amino acid by DNA differed from that by RNA.

In order to place the difference in the degree of binding of various amino acids by RNA on some quantitative basis, the phenomenon was investigated under conditions of an entire range of amino acid concentration, and also with varying amounts of RNA in the reaction mixture. It should be remembered that in all previous experiments the amino acids were present only in trace amounts. The % binding of these amino acids by RNA remained constant, more or less, throughout; of the total label present, about 3% was bound in the case of glycine, while with tyrosine the value, which showed a somewhat higher degree of variation, rarely exceeded 1.5%.

In order to determine the maximal "capacity" of RNA to bind glycine, the labeled tracer was diluted with "cold" glycine and varying amounts of this were added to the incubation mixtures containing DNA. The results are shown in Table 4.

It will be seen from Table 4 that over a wide range of concentrations, the degree of glycine binding by RNA was proportional to the concentration of the former. The limit of this concentration effect was not reached when glycine reached saturation concentrations. Although the relative values (percent bound) varied

TABLE 4.
The Effect of Glycine Concentration on Its Binding by RNA.

Glycine concentration		% bound in expts.				
μ moles	mg.	1	2	3	4	5
0,125	0,009	3,3	—	—	—	—
1,0	0,075	2,9	—	—	—	—
10,0	0,75	3,1	3,1	—	—	2,5
20,0	1,5	2,8	2,3	—	—	—
100,0	7,5	—	2,7	—	1,6	2,2
200,0	15	—	2,3	3,4	1,6	—
500,0	37,5	—	2,1	2,7	1,7	—
1000,0	75	—	—	2,8	1,4	2,1
2000,0	150	—	—	2,3	—	—
6000,0	450	—	—	—	—	1,7

somewhat from experiment to experiment, there was no demonstrable effect of the amount of carrier present on these values.

Similar experiments were also carried out with tyrosine. The latter was only investigated over a narrow concentration range because of its poor solubility. Within the range investigated the proportion of tyrosine present, which was bound by RNA was not affected by tyrosine concentration.

It was thus not possible, under the conditions employed, to find concentrations of amino acids sufficiently high to "saturate" the RNA in the amounts used. While in the case of tyrosine this may have been due to its low solubility and the consequent low limiting concentrations of this amino acid in the reaction mixture, this explanation could hardly apply to glycine which, because of its high solubility, could be added to the reaction mixture in high concentrations.

This circumstance raised certain doubts about the true nature of the binding of glycine by RNA, and this was checked in two ways, as follows: 1) the purity of the tracer was checked by chromatography, and 2) the estimate of the amount of glycine bound, obtained from the radioactivity values, was checked by a direct specific chemical method. It could be argued that some radioactivity, not identical with glycine, was bound by the RNA, and was therefore unaffected by the carrier glycine added.

Chromatography of the tracer glycine in butanol–acetic acid solvent system revealed the presence of one only ninhydrin-reactive spot, corresponding in position to glycine. Scanning of the chromatograms revealed that the entire radioactivity was present in that position also. This suggested that the labeled glycine employed contained no other tracer impurities which may have been bound by the RNA and created an artefact suggesting the actual binding of glycine. This was also confirmed by the quantitative glycine determinations according to Alexander [19]. Control experiments showed that during distillation with ninhydrin the RNA preparation used did not liberate formaldehyde or any other substances interfering with the reaction with chromotrophic acid. The chemical analysis accounted for 83-86% of the glycine presumed bound from the determination of bound radioactivity. The bound radioactivity was therefore not an artefact, and was actually a measure of true binding of glycine.

The effect of RNA concentration on the binding of tyrosine and glycine was also investigated. The amount of RNA present in the reaction mixture was varied, while the amount of labeled amino acid present was maintained constant. Althought the actual relative amount of radioactivity (and therefore amino acid) increased with increasing RNA concentration (Table 5), there was no proportional relation between the amount of RNA present and the proportion of glycine or tyrosine bound.

TABLE 5.
The Effect of RNA Concentration on Its Binding of Glycine and Tyrosine.

RNA present, mg	% bound in expts.			
	1	2	3	4
	C^{14}-glycine			
5	1,6	2,3	1,4	
10	3,2	3,1	2,3	
20	2,3	3,7	2,8	
30	2,1	5,1	3,4	
	C^{14}-tyrosine			
5	1,5	—	0,6	0,6
10	2,8	—	1,5	0,8
20	5,6	—	2,1	1,5
30	7,2	—	2,6	2,2

DISCUSSION

It follows from the present work that the binding of amino acids is not confined to proteins, since the phenomenon occurred even more strongly with nucleoprotein and with nucleic acids. The binding of amino acids by starch and glycogen was not permanent and was probably due to physical adsorption.

Treatment of nucleoprotein complexes with hot TCA showed that amino acids bound by these polymers were attached to the protein moiety. Treatment of the nucleoprotein-amino acid complex with either alkali or ninhydrin failed to remove anything but a small proportion

of the bound label, thus proving that it was bound to the protein in the same way as it was shown to be bound after the incubation with protein alone. Nucleic acids bound 30-40 times the label bound by the same weight of protein, but the major proportion of the label could be removed by alkaline or ninhydrin treatment.

Nucleic acids were thus found capable of binding large amounts of amino acids when present alone, while in combination with proteins the nucleic acid moiety enhanced amino acid binding by the protein of the complex. The activation of amino acid binding by proteins upon addition of nucleic acids was also reported by Kritsman and Sukhareva [8]. It is possible that the ability to bind amino acids strongly is related to some role of the nucleic acids in protein synthesis, although it is not suggested that this is their exclusive role in this process.

The wide variation of the values obtained with the same reaction mixture compositions in different experiments cannot be overlooked. The reasons for such variation are not clear, but it should be mentioned that it has been reported by other authors [12, 13] working with proteins, and that in biological systems radioactive label incorporation values generally vary through a series of experiments carried out under identical conditions.

Our present results do not allow any firm conclusions about the exact nature of the bond formed between the amino acids and the proteins or nucleic acids. Measures sucy as ninhydrin treatment or re-solution in alkali followed by reprecipitation while being capable of removing the bulk of adsorbed radioactivity from RNA or DNA, caused only slight loss of activity bound by proteins. The type of treatment employed is capable of disrupting not only adsorption complexes, but also phosphate bonds and hydrogen bonds. Attempts to demonstrate hydroxamate formation under the conditions of our experiments, in the presence of amino acids, nucleic acids, and hydroxylamine, were unsuccessful. The binding of amino acids by nucleic acids was, apparently, of some specific nature, and its mechanism remains obscure.

Since all the present experiments were carried out with commercial samples of RNA and DNA, which were no longer in their native state and consisted of mixtures of polynucleotides in various states of polymerization, it was of interest to repeat some of the experiments with highly polymerized samples of nucleic acids. Highly polymerized RNA and DNA prepared from rat liver by extremely mild methods [16], were found to have nearly excatly the same ability to bind amino acids as did the commercial samples. This clearly indicated that the preservation of the native state of the nucleic acid, and the number of free terminal groups, were of little importance in the phenomenon studied.

The high capacity of nucleic acids, proteins and, particularly, of nucleoproteins to bind amino acids suggests that to be a property related to biological systhesis of proteins, and thus deserves further detailed study. There are, however, no indications that the isolated preparations in any way simulated the reactions of protein synthesis. Such supposition is excluded by the observations that various denatured protein preparations bound amino acids in approximately the same intensity, by the absence of a proportional relationship with incubation time and by the complete absence of the slightest evidence for net synthesis of any kind of protein.

SUMMARY

Proteins, nucleoproteins, nucleic acids and some other biological polymers have been shown to have the ability to bind amino acids in aqueous solutions.

RNA and DNA preparations have been shown to bind 30-40 times, and RNP and DNP preparations, 3-8 times the amount of amino acids bound by an equal weight of protein. The protein moiety of natural nucleoproteins bound labeled amino acids as strongly as proteins alone. Nucleic acids were found to lose the bulk of bound labeled amino acids when reprecipitated from alkaline solutions or if treated with ninhydrin. Starch and glycogen could be made to part with all adsorbed amino acids by simple washing with TCA or with acid alcohol.

The amount of amino acids bound by nucleic acids was found to be practically directly proportional to amino acid concentration and was not affected by salt concentration or by variation in pH over a wide range.

It appears that the nature of the bond formed between amino acids and proteins is different from that formed with nucleic acids. The nature of the phenomenon was discussed and it was suggested that, although it may bear relation to protein synthesis, it did not actually simulate that process.

LITERATURE CITED

[1] H. Tarver in: The Proteins 2, ed. by H. Neurath and K. Bailey (Acad. Press, New York, 1955), p. 26.

[2] M. Rabinovitz, M.E. Olson, and D.M. Greenberg, J. Biol. Chem. 210, 837 (1954).

[3] R. Brunish and J.M. Luck, J. Biol. Chem. 197, 869 (1952).

[4] M.G. Kritsman, A.S. Konikova and Ts.D. Osipenko, Biokhimiya 17, 488 (1952).

[5] B.A. Askonas, J.L. Simkin, and T.S. Work, IV Intern. Congress of Biochemistry, Symposium No. VIII, Reprint No. 1 (1958).

[6] V.G. Allfrey, A.E. Mirsky and S. Osawa, Nature 176, 1042 (1955).

[7] I.B. Zbarskii and K.A. Perevoshchikova, Voprosy Med. Khim., 6, 34 (1960).

[8] M.G. Kritsman and B.S. Sukhareva, Proc. All-Union Meeting of the Socy. Physiol., Biochem and Pharm. Vol. 2, 147, (Minsk, 1959).

[9] A.S. Konikova, M.G. Kritsman, and O.P. Samarina Biokhimiya 19, 440 (1954).

[10] O.P. Samarina, M.G. Kritsman and A.S. Konikova Biokhimiya 21, 10 (1956).†

†Original Russian pagination. See C.B. Translation.

[11] A.G. Pasynskii, M.S. Volkova and V.P. Blokhina, Doklady Akad. Nauk SSSR 101, 317 (1955).††

[12] S.E. Shnol', Voprosy Med. Khim. 1, 327 (1955).

[13] D.G. Cornwell and J.M. Luck, Arch. Biochem. and Biophys. 73, 391 (1958).

[14] A.S. Konikova and M.G. Kritsman, Voprosy Filisofii 1, 143 (1953).

[15] I.B. Zbarskii and G.P. Georgiev, Biokhimiya 24, 192 (1959).†

[16] G.P. Georgiev, Biokhimiya 24, 472 (1959).†

[17] B.I. Zbarskii, I.B. Zbarskii and A.I. Solntsev: Handbook of Biological Chemistry [in Russian] 2nd Ed. (Medgiz, Moscow, 1954)

[18] I.B. Zbarskii and K.A. Perevoshchikova, Biokhimiya 22, 295 (1957).†

[19] B. Alexander, G. Landwehr, and A.M. Seligman, J. Biol. Chem. 160 , 51 (1945).

[20] G. Zubay and P. Doty, Biochim. et biophys. acta 29, 47 (1958).

[21] C.D. Jardetzky, J. Am. Chem. Soc. 80, 1125 (1958).

†† See English translation.

VARIABILITY OF P/O RATIOS IN OXIDATIVE PROCESSES

V.P. Skulachev and L.L. Kiselev

Department of Animal Biochemistry, M.V. Lomonosov
State University, Moscow
Translated from Biokhimiya Vol. 25, No. 3, pp. 452-458,
May-June, 1960
Original article submitted August 20, 1959

In a previous communication [1] results of a study of in vitro regulation of two oxidation pathways—one associated with phosphorylation and one independent of phosphorylation—were presented. It was established that isolated pigeon liver mitochondria respond, by repeated shifts from one pathway to the other, to fluctuations in ATP content, i.e., the relationship of the two pathways is variable and can be regulated.

The study of the variability in the relationship of these two pathways as it depends on factors acting in vivo and related to the condition of the animals is the subject of this communication.

METHODS

Oxidative phosphorylation of liver mitochondria was measured by a previously described method [1] at various times in the year using pigeons in different physiological conditions. The mitochondria, which had been isolated in 0.25 M sucrose, were incubated 20 minutes at 26° in air. The reaction mixture (2 ml) contained: potassium phosphate (pH 7.4), 40 μ moles; KCl, 100 μ moles; glucose, 150 μ moles; hexokinase (fifth purification stage according to Berger et al. [2]) in amounts three times greater than those necessary for maximal phosphorylation rates; mitochondria from 300-400 mg of liver in 0.25 M sucrose; potassium succinate (pH 7.4), 50 μ moles; $MgCl_2$, 10 μ moles; AMP, 3 μ moles.

In most experiments P/O ratios were measured immediately after isolation of mitochondria and also after preincubation of mitochondria in the absence of substrate. Mitochondria were preincubated on a shaker for 10-15 or 25-30 minutes at 26° in a solution containing the first five components of the reaction mixture.

RESULTS

In experiments from November, 1958, to April, 1959, on the oxidation of succinic acid by pigeon liver mitochondria, significant variations in the P/O ratio were noted. These variations were sharply magnified in mitochondria which had been preincubated in the absence of substrate. The maximum difference in P/O ratios obtained with mitochondria which had not been preincubated was 0.6 (ratios from 1.1 to 1.7), while that obtained with mitochondria which had been preincubated 25 minutes was 1.6 (ratios from 0 to 1.6).

As Table 1 shows (average monthly P/O ratios given), the probability that differences in P/O ratios are fortuitous is small where measurements were made without a preincubation, and where there was a preincubation it is even smaller ($p < 0.011$).

Dynamic changes in the P/O ratio for a single month are shown in Fig. 1. Results of all experiments in March are shown in the graph , and for comparison ratios obtained in February and April are also shown. In the majority of experiments, ratios were measured under three different conditions—without preincubation, after a short preincubation, and after a long preincubation. The March series of experiments is characterized by a shift to a nonphosphorylating oxidation pathway (NO), as shown by the reduction in P/O ratios. Figure 1 shows that the tendency to this shift is already evident at the

TABLE 1. Average Monthly P/O Ratios

Month	Measurement, no PR*			Measurement, 25 min. PR*		
	P/O	number of expts.	p	P/O	number of expts.	p
November	1,1 ±0,04	10		0	4	
December	1,7 ±0,08	5	0,001	1,6±0,1	3	<0,001
January	1,4 ±0,08	8	0,029	Not measured		
February	1,3 ±0,04	8	0,3	0,95±0,04	7	0,008
March	1,1 ±0,04	8	0,001	0,3 ±0,09	10	<0,001
April	1,35±0,01	4	0,026	0,8 ±0,09	4	0,011

*PR = preincubation. Note: Average arithmetical values and average errors; p—probability that the difference in the P/O ratio of a given month from that for the preceding month is fortuitous.

end of February, and after a long preincubation (curve 3) the reduction in P/O ratio at this time is pronounced. In the experiment of March 6, this reduction becomes clear with a short preincubation as well (curve 2). In the experiments of March 11, 12, and 13, the shift toward a nonphosphorylating oxidation pathway is clear even in the samples which have not been preincubated (curve 1). At this time the shift away from a phosphorylating pathway is complete in those samples subjected to a long preincubation. After March 13, the ratios obtained under each of the three treatments begin to rise. It is obvious that in the March series of experiments changes in P/O ratios are most pronounced in samples preincubated for a long period. This pattern of change for one month (Fig. 1) is analogous to that for a period of months (Table 1), i.e., changes are most pronounced where there has been a preincubation in the absence of substrate.

In analyzing this phenomenon we investigated the effect on it of ATP, the content of which plays a substantial role in the shift in oxidation pathways during preincubation [1, 3]. It was shown that the variability in the P/O ratio which is enhanced by preincubation is masked if ATP is present in the preincubation mixture.

As shown in Table 2, a 25-minute preincubation period in the experiments of February 20 and 24 causes a shift toward a nonphosphorylating oxidation pathway, while the same treatment from January 20 to February 13 has no such effect. The shift in P/O ratio with time of year escapes detection if there is no preincubation period or if preincubation is carried out in the presence of catalytic amounts of ATP (final concentration 2 μ moles per sample).

With preincubation in the presence of ATP, the P/O ratio is maintained at its original level, while in the absence of ATP there was a complete uncoupling of oxidation and phosphorylation, as shown by Fig. 2. The uncoupling observed at about 90 minutes in the sample preincubated with ATP is due to utilization of the ATP by hexokinase present in the preincubation medium, since new additions of ATP can prevent uncoupling and maintain oxidative phosphorylation for a period of several hours. Such a prolonged preincubation period leads ultimately to a reduction in oxidation activity, but the residual oxidation is associated with an unchanged level of phosphorylation, or even, in some cases, with a somewhat higher level of phosphorylation.

TABLE 2. ATP as a Factor Preventing a Change in P/O Ratio with Preincubation

Date of expt.	P/O before preincubation	P/O after 25 minutes preincubation	
		no ATP	ATP
Jan 20-Feb 13	1,25	1,2	—
Feb 20	1,3	0,75	1,45
Feb 24	1,25	0,45	1,25

Figure 3 shows that preincubation of mitochondria without ATP is accompanied by a fall in the optical density of the suspension at 520 mμ, which is an indication of mitochondrial swelling. ATP impedes this fall markedly, the duration of its effect being proportional to its concentration in the preincubation mixture. Therefore there is reason to suppose that in preventing swelling, ATP retards the shift to nonphosphorylating oxidations.

DISCUSSION

The data obtained on the periodic variability of the P/O ratio in isolated pigeon liver mitochondria may be

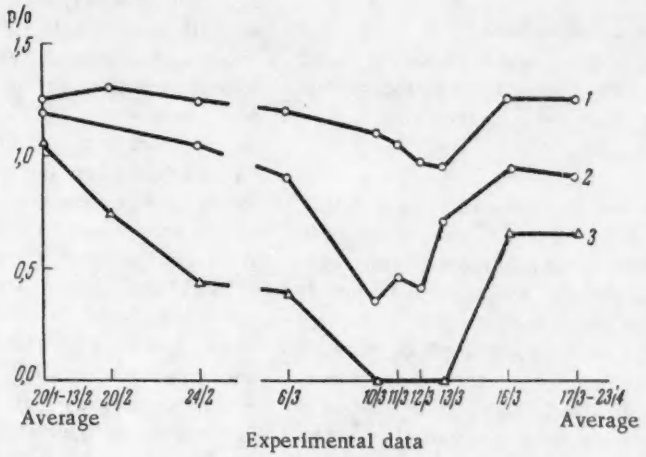


Fig. 1. Dynamics of the P/O ratio for March. 1) Without preincubation; 2) after a short preincubation (11 minutes); 3) after a long preincubation (25 minutes).

regarded as an indication of a variability in the relative amounts of coupled and uncoupled oxidations in the organism.

These changes in P/O ratios cannot be explained by procedural factors or by a fortuitous ordering of the data; they are the result of the action of certain factors related to the condition of the organism. An analysis of these factors and a study of the in vivo regulation of oxidation pathways requires further investigations. Seasonal factors might be considered as among the most probable causes of this variability. The shift to a phosphorylating pathway at the beginning of the winter occurred at the time of the completion of fall moulting, and the shift to a nonphosphorylating pathway at the end of February corresponded with the beginning of the spring moulting. It is highly probable that external temperatures play a role, the more so since it has been recently shown that there is a change in the relative roles played by coupled and uncoupled oxidations which is associated with thermoregulated behavior [4-6].

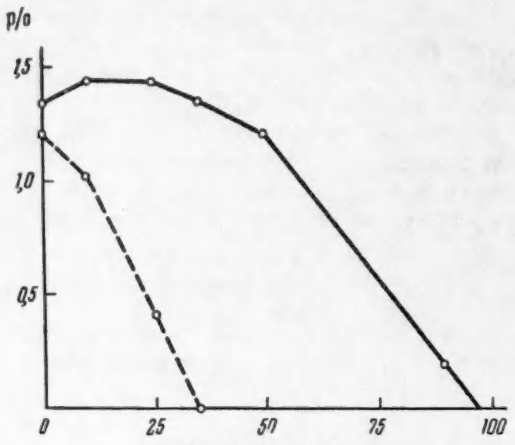


Fig. 2. Inhibition of the uncoupling effect of preincubation by ATP. Dotted line—preincubation without ATP; solid line—preincubation with ATP (2 μ moles per sample).

Of interest is the fact that the variability of P/O ratio in dependence on the condition of the organism is manifested more clearly under conditions in which uncoupled oxidations are flavored than under those usually selected to study oxidative phosphorylation. As Beyer, Lŏw, and Ernster [7] have noted, the methods of studyin oxidative phosphorylation are beingconstantly improved with a view to increasing the P/O ratio. The method of measuring the P/O ratio commonly employed at present favors coupled oxidations at the expense of uncoupled oxidations. Results obtained by this method therefore reflect to a greater degree the maximal capacity of a given oxidative system to carry out coupled oxidations rather than the actual physiological relationship of the phosphorylating and nonphosphorylating pathways. It is sufficient to point out that several components of the reaction mixture used in experiments on oxidative

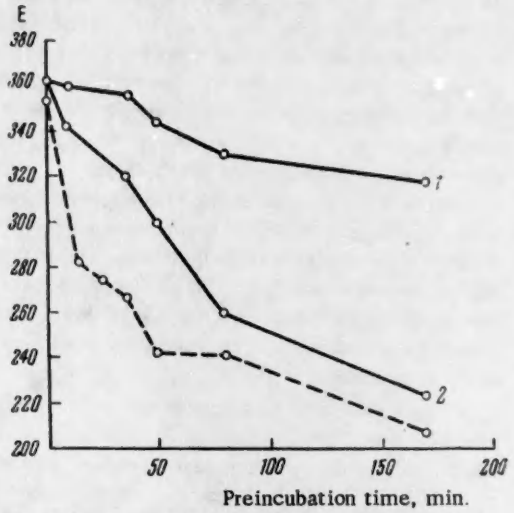


Fig. 3. The stabilization of the optical density of a mitochondrial suspension by ATP. Dotted line—preincubation without ATP; solid lines—preincubation with ATP at: 1) 2 μ moles per sample; 2) 0.5 μ mole per sample

phosphorylation promote a shift from nonphosphorylating pathways to phosphorylating pathways. Among these are magnesium ions, inorganic phosphorus and ADP (AMP) in excess, and the hexokinase—glucose system, which has a trapping function [8]. In adddition, ATP, which is also a stimulator of oxidative phosphorylation [1, 3, 9, 10], is often introduced into the reaction mixture. With respect to substances which promote a shift from phosphorylating to nonphosphorylating pathways, they are certainly not added to the reaction mixture, and, indeed, attempts are made to remove them from the reaction mixture if possible. For example, versene removes calcium ions from the mitochondria.

In the experiments described in this communication, we have excluded from the reaction medium and the isolation medium such promoters of oxidative phosphorylation as versene and ATP; this has evidently facilitated the demonstration of a variability in P/O ratios. A still more radical means of demonstrating such variability was the preincubation of the mitochondria without substrate. It is well known that calcium ions, which activate nonphosphorylating pathways, render changes in P/O ratios under the influence of thyroid and parathyroid hormones (experiments of Yang Fu-yü in our laboratory, studies of Ernster et al. [7], Dawson and Jones [15]) more pronounced.

The method used for measuring P/O ratios should obviously be determined by the purpose of the study. If

the purpose is to obtain the maximum P/O ratio of which the given system is capable, then it is appropriate to use ordinary methods of measuring oxidative phosphorylation, these being methods which selectively activate phosphorylating pathways. If, however, the purpose is to determine the actual physiological roles played by the two pathways, then it is desirable to employ activators of nonphosphorylating pathways together with those of phosphorylating pathways, with which it is indeed impossible to dispense. If additional activators of phosphorylating pathways are introduced into a system which is already balanced with respect to activators of the two pathways, the equilibrium is shifted in the direction of phosphorylating pathways and the relative roles played by the two pathways are once more obscured. In our experiments this occurred when ATP was added to the preincubation medium. In the presence of this activator of phosphorylating pathways, preincubation was without effect in rendering manifest the variability of the P/O ratio, since it remained high regardless of the length of the preincubation period.

The parallelism between the action of ATP in inhibiting uncoupling and in inhibiting mitochondrial swelling once more indicates the interrelation of the ratio of phosphorylating to nonphosphorylating oxidations and mitochondrial structure [10-13]. Here ATP is an energy donor for the maintenance of the phosphorylating structure, and it might be expected that not ATP as such, but the process of its breakdown, is the immediate cause of the shift to phosphorylating oxidation pathways. The presented hypothetical scheme illustrates the system under discussion.

The activators of phosphorylative oxidation (PO) and the activators of nonphosphorylative oxidation (NO) are shown, and the mechanism of action of one of them (ATP) is illustrated. The role of ATP as an activator of phosphorylative oxidation resides in its function as a substrate of mitochondrial ATPase (an enzyme studied by Lehninger [14]), which converts mitochondria from the swollen condition to a shrunken condition.

The shrinking of the mitochondria is accompanied by the emergence of a capacity for PO, i.e., for the formation of ATP in association with hydrogen transfer through the respiratory chain. This scheme illustrates the hypothesis that ATP is not only a product, but also a stimulator of phosphorylative oxidation. It is still unclear which of the factors in the scheme are physiological regulators of oxidative pathways, but it is incontrovertible that they all affect P/O ratios in vitro.

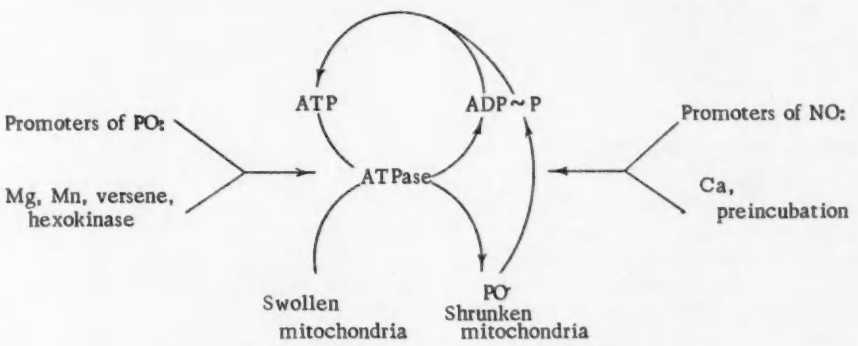
The P/O ratio in vitro is therefore determined by two groupsof factors—regulators of phosphorylative and nonphosphorylative oxidation which act inside the organism and which determine the state of the isolated mitochondria, and regulators which act in vitro, such as the components of the isolation medium and the reaction medium (In our case these were ATP, magnesium, hexokinase). Preincubation was also a factor which made possible the demonstration of P/O variability.

SUMMARY

The periodic variability in the P/O ratio of pigeon liver mitochondria as it depends on the physiological condition of the organism was investigated. Such variability was demonstrable if, in the measurement of P/O ratios, activators of nonphosphorylative oxidation (preincubation of mitochondria in the absence of substrate) were used as well as activators of phosphorylative oxidation. The effect of preincubation was not manifest if ATP was present, since this compound prevented the activation of nophosphorylative oxidation and inhibited mitochondrial swelling during the preincubation period.

We wish to thank Professor S.E. Severin for his valuable suggestions and help during this study.

Scheme of Regulation of the Two Oxidation Pathways in Vitro



Note. PO—phosphorylative oxidation; NO—nonphosphorylative oxidation; ~P— high-energy bond formed during oxidative phosphorylation.

LITERATURE CITED

[1] V.P. Skulachev and L.L. Kiselev, Biokhimiya 25, 90 (1960).*

[2] L. Berger, M.W. Slein, S.P. Colowick, and C.F. Cori, J. Gen. Physiol. 29, 379 (1946).

[3] L. Ernster and H. Low, Exptl Cell Res., Suppl. 3, 133 (1955).

[4] S. Panagos, R.E. Beyer, and E.J. Masoro, Biochim. et biophys. acta 29, 204 (1958).

[5] E.J. Masoro and J.M. Felts, J. Biol. Chem. 234, 198 (1959).

[6] R.E. Smith and A.S. Fairhurst, Proc. Natl. Acad. Sci. 44, 705 (1958).

[7] R.E. Bever, H. Löw, and L. Ernster, Acta chem. scand. 10, 1039 (1956).

[8] S.E. Severin, V.P. Skulachev, and L.L. Kiselev, Doklady Akad. Nauk SSSR 128, 628 (1959).

[9] L. Ernster and O. Lindberg, Physiol. Rev. 20, 13 (1958).

[10] O. Lindberg and L. Ernster, Nature 173, 1038 (1954).

[11] A.L. Lehninger, Harvey Lectures 49, 176 (1955).

[12] V.P. Skulachev, Usp. Sovremennoi Biol. 46, 241 (1958).

[13] R.E. Beyer, L. Ernster, H. Löw, and T. Beyer, Exptl.Cell. Res. 8, 586 (1955).

[14] A. L. Lehninger, R.L. Ray, and M. Schneider, J. Biophys. and Biochem. Cytol. 5, 97 (1959).

[15] J. Dawson and E.A. Jones, Intern. congr. clin. chem. (Stockholm, 1957), p. 86.

*Original Russian pagination. See C.B. Translation.

THE ABSORPTION BY FISH OF INORGANIC CARBON FROM THE SURROUNDING MEDIA, AND ITS PARTICIPATION IN METABOLISM

K.F. Sorvachev and O.V. Belokopytova

Isotope Laboratory, Department of Biology and Soils,
the M.V. Lomonosov State University, Moscow
Translated from Biokhimiya Vol. 25, No. 3, pp. 459-464,
May-June, 1960
Original article submitted August 20, 1959

It was assumed up to recent times that carbon dioxide represents the end-product in the process of living cell dissimilation, and represents a substance which is useless and even harmful for heterotrophic organisms. It was also assumed that the animal organism is unable to assimilate carbon dioxide and to use it for the synthesis of organic substances.

However, as shown by recent investigations, such a point of view proved to be erroneous. By using new, more precise methods of investigation (chromatography, labeled atoms, etc.) it was demonstrated that all living organisms, from bacteria to man, possess the ability to assimilate carbon dioxide. The difference between the autotrophic and heterotrophic organisms is rather of quantitative than qualitative character [1, 2]. At present there are sufficient publications in the literature which show the possibility of carbon dioxide assimilation by the tissue of various organs, its incorporation into protein, carbohydrates, fat, and other compounds. Such investigations were conducted in vivo as well as in vitro, but mostly on mammals [3 - 14]. It would be of interest to follow up the process of absorption of inorganic carbon from the water by fish, its distribution in organs and tissues, as well as its incorporation in various organic compounds of the organism.

METHODS

Into an aquarium of 2.5 liter capacity with water there was added carbon-tagged sodium carbonate ($Na_2C^{14}O_3$) in an amount which would produce an initial radioactivity of the water in the aquarium within the range of $1.6 \cdot 10^6$ - $3.6 \cdot 10^6$ disintegrations per minute/ml. The fish (crucians and carp of 20 - 40 g weight) were kept in the radioactive medium for 24, 48, and 72 hr. During the experiment the fish were not fed. Air was not bubbled through the solution, to avoid loss of radioactive carbon in the form of $C^{14}O_2$. During the experiment, water from the aquarium was sampled for radioactivity determination; simultaneously, we determined the carbonates, bicarbonates, carbon dioxide and the pH of the medium [15]. After a certain lapse of time the fish were taken out of the aquarium, washed with tap water, weighed and dissected. Radioactivity determinations were made on organs and tissues (liver, brain, intestine, bone, kidney, blood, eyes, heart, spleen, muscle, skin, gills and bile). The blood was taken from the heart by means of Roux's sterile micropipette. The excised organs were rinsed from blood by tap water, dried, ground to a powder, and defatted. Dry batches of 2 to 10 mg were examined on a B-2 mounting with an end-window counter. The proteins from the homogenate of muscle, liver and intestine were precipitated by 5% trichloroacetic acid (4 volumes of trichloroacetic acid per gram of raw tissue defatted), and brought to constant weight. The dry protein batches were tested for radioactivity. The glycogen from the liver and muscles was precipitated from trichloroacetic acid by alcohol [16]. The fat was extracted in a Soxhlet apparatus. The radioactivity of organs, tissues, proteins, glycogen, and fat were expressed as the amount of disintegrations per minute, per gram of dry weight. The amino acids of the extracts, freed from the trichloroacetic acid by means of an aqueous solution of ethyl ether, were determined chromatographically according to Bode [17]. Then, the radioactivity of the spots was determined by means of an end-window counter [18]. The serum proteins of the fish were separated into fractions by means of paper electrophoresis [19]. The staining of the strips and the removal of the color was conducted according to the directions of Juncks, Jetton, and Durrum [20]. The quantitative study of the electrophoregrams was conducted by elution with a subsequent colorimetric analysis of the eluate. Simultaneously, determinations were made of the radioactivity of the separate protein fractions on paper and in the eluate.

RESULTS

Changes in Radioactivity of the Medium and the Distribution of C^{14} in the Organs and Tissues of Fish. A sharp decline in the radioactivity of the water is already taking place during the first hours of the fish remaining in the aquarium containing $Na_2C^{14}O_3$ (Table 1).

Within 28 hours the radioactivity of the water has decreased almost five times, but during the second and

TABLE 1. Changes in the Radioactivity of Water and its Bicarbonate and CO_2 Content in Experiments with Carp

Time, hr	Radioactivity of medium desintegr/min/ml water	Bicarbonates, mg/liter	Free CO_2, mg/liter	pH
Prior to expt.	1600000	118,50	—	8,3
1	1500000	116,00	3,45	
4	1220000			
24	500000	117,00	3,70	6,8
28	383000			
48	300000	117,04	3,80	6,8
52	280000	119,50	3,95	
72	270000	120,73	4,19	6,6

third days the level of radioactivity has remained almost constant. If one assumes that the decline of C^{14} in the water is related mainly to the deposition of the isotope in the fish organism, it would mean that in 24 hours a carp of 20-30 g body weight absorbs, on the average ,70% of the initial radioactivity, and a crucian absorbs 60% of same. Within 48 and 72 hours, there were deposited in the body of the carp 80 and 83% resp. of radioactivity, and in a crucian 74 and 70% resp. of same. Some portion of the radioactive isotope has been unquestionably used up by bacteria, as well as lost from the solution into the air in the form of $C^{14}O_2$. However, in control experiments (when there were no fish in the aquarium) we were unable to establish any marked decrease in radioactivity.

These experiments gave some reason to assume that fishes are able to absorb from the surrounding media large quantities of inorganic carbon. The latter would undoubtedly enter in the fish organism into complex exchange processes. This assumption was quite conclusively confirmed in our subsequent investigations.

A considerable amount of C^{14} was found in all of the analyzed organs and tissues of fish. The radioactivity in the body of fish increases with the time of their stay in the radioactive medium, as well as with the isotope content of the medium (Tables 2 and 3). The highest radioactivity was found in the blood serum, liver, intestine, bone, kidney, brain tissue, eyes, gills, and heart; of lesser activity are the muscles, skin, bile, and red blood cells.

The Radioactivity of Proteins, Glycogen and Fat.

The experiments on the rate of C^{14} penetration into proteins, glycogen and fat of the liver, muscles and intestine were conducted on carp at minimum radioactivity of the medium (Table 4). It was established that during the first day of exposure the C^{14} penetrates foremost into the liver proteins (1055 disint /min per mg of dry protein), less into the glycogen (800 disint /min per mg of glycogen) and fat (870 disint /min per mg of fat). But the picture undergoes a drastic change during the second and third days of exposure. On the third day the radioactivity of the liver proteins has increased three times, of glycogen, eight times, and of fat, over two and one half times.

TABLE 2. Distribution of C^{14} in the Organs and Tissues of Crucians (disint/min per mg of dry weight). Radioactivity of the medium $3.5 \cdot 10^6$ disint/min per ml.

Tissues	24 hrs	48 hrs	72 hrs
Liver	10000	13200	14340
Brain	9480	13140	13330
Intestine	9380	12030	10250
Bone	5500	11040	9770
Kidneys	9350	11230	10340
Blood	8520	12030	10710
Blood serum*	185000	320000	10710
Red blood cells	660	750	850
Eyes	9320	10057	8770
Gills	5300	10050	9250
Heart	6440	10030	8800
Spleen	5880	9475	8657
Bile	4500	5370	4240
Muscles	2000	2230	1540
Skin	2000	2340	1050

*The serum radioactivity (disint/min) is calculated per 0.1 ml.

TABLE 3. Distribution of C^{14} in the Organs and Tissues of Carp (disint/min per mg of dry weight) Radioactivity of the medium $1.6 \cdot 10^6$ disint/min per ml

Tissues	24 hrs	48 hrs	72 hrs
Liver	3080	3730	6580
Brain	2170	2290	3240
Intestine	1710	3090	2880
Bone	3450	3970	4760
Kidneys	1260	1560	2800
Blood	1300	3090	2800
Blood serum†	14880	18200	18400
Red blood cells	250	880	1200
Eyes	1430	1500	1680
Gills	1970	2810	2640
Heart	730	1565	2640
Spleen	610	1040	1680
Bile	217	561	920
Muscles	530	760	800
Skin	500	1000	920
Scales	4820	6440	7000

†The serum radioactivity (disint/min) is calculated per 0.1 ml.

TABLE 4. Distribution of C^{14} in Proteins, Glycogen and Fat of the Liver, Muscles and Intestine of Carp (desint/min mg of dry weight). Radioactivity of the medium 1 6 · 10^6 disint/min per ml

	24 hr.			48 hr.			72 hr.		
	liver	muscles	intes-tine	liver	muscles	intes-tine	liver	muscles	intes-tine
Protein	1055	87	439	1565	439	913	3440	640	1160
Glycogen	800	640	—	2058	16120	—	6480	12880	—
Fat	870	192	304	1520	280	400	2400	400	560

The picture of C^{14} penetration into the proteins, glycogen and fat of the muscles is somewhat different. At the end of the first day of exposure the radioactivity of the muscle proteins constitutes only 87 disint /min per mg of dry weight, of glycogen, 640, of fat, 192 disint /min per mg of dry weight. In the course of two days the radioactivity of muscle proteins increases five times, of glycogen, 20 times (16200 disint /min per mg), of fat, one and one half times. During the third day of exposure the radioactivity of the proteins and fat continue to increase, while the radioactivity of the muscle glycogen drops slightly, while remaining still high (12880 disint /min per mg), being twice as high as the radioactivity of liver glycogen.

We were unable to isolate glycogen from the intestine. The radioactivity of the proteins and fat was comparatively small while increasing with the time the fish stayed in the radioactive medium. This may presumably be explained by the fact that during the second and third days of fasting, the synthesis of glycogen in the muscles of fish predominates over the synthesis of glycogen in the liver. On the other hand, it is known that the breakdown of glycogen in the liver proceeds at a pace which is several times more intensive than in the muscles.

Incorporation of C^{14} into Some Amino Acids.‡ Some amino acids from liver, muscle, and intestine extracts of the experimental fish possessed radioactivity (Table 5). The highest radioactivity was exhibited by glycine, cystine, leucine, valine, lysine, and by glutamic and aspartic acids of the liver. The highest radioactivity was possessed by the cystine of the muscles and intestine, the tryosine and arginine of the liver, and the lysine of the intestine. Histidine and triptophane exhibited no radioactivity at all; the activity of α-alaine was insignificant in all of the extracts under study.

Incorporation of C^{14} into the Protein Fractions of Blood Serum. Preliminary investigations disclosed that blood serum posesses a high degree of radioactivity (see Tables 2 and 3). Towards the end of the first day exposure to radioactive water, the radioactivity of 0.1 ml of carp's blood serum constitutes 14.8 thousand disint/min; during the second and third days, 18.2 and 18.4 thousands disint /min. A study of the electrophorogram of the serum proteins showed that the highest radioactivity is possessed by the alpha-and beta-globulins and the albumins. The radioactivity of the gamma-globulins (disint /min/per g of serum protein) is insignificant .

Protein fraction	Duration of expt., hrs		
	24	48	72
γ-globulins	Bkg.	140	200
β-globulins	120	560	920
α-globulins	160	600	1280
Albumins	120	480	760

DISCUSSION

On the basis of our experimental results one can state definitely that fishes are assimilating carbon from mineral compounds dissolved in water. The absorption of C^{14} is here increasing with the length of exposure to the radioactive medium and with the concentration of the isotope therein.

The absorbed C^{14} accumulates in substantial quantities in the blood serum, liver, bones, scales, brain, intestines, and kidneys, and in lesser quantities in bile and muscles. The fact that C^{14} is present in the carbohydrates, proteins and fat is an indication that the carbon absorbed by fish from the surrounding media is taking an active part in the metabolism of the organism, and, after transformations have taken place, it is being converted into organic compounds.

In our observations on chromatograms we were able to establish the radioactivity of the spots of certain amino acids, which were isolated from liver, muscle, and intestinal extracts. If this phenomenon is not to be considered as being an artefact and that the labeled carbon which has been fixed in the chromatograms belongs to the amino acids and not to the accompanying impurities, it could aid in the study of the biological synthesis of

‡ While radioactivity of amino acid spots in chromatograms does not represent an absolute proof of the incorporation of a labeled element in the respective amino acid (see Discussion), it nevertheless warrants such an interpretation as most probable. Therefore, we allowed ourselves to use the terms "incorporation of C^{14} into amino acids" and "radioactivity of amino acids" in relation to the radioactivity of amino acid spots in chromatograms.

TABLE 5. Incorporation of C^{14} Into Some Free Amino Acids of Extracts of Organs and Tissues of Carp

Amino acids	Radioactivity of media 1.6 · 10^6 disint/min/ml								Radioactivity of media 2.5 · 10^6 disint/min/per mg					
	liver			muscles		intestine			liver		muscles		intestine	
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	24 hrs	48 hrs	72 hrs	48 hrs	72 hrs	48 hrs	72 hrs	48 hrs	72 hrs
Cystine	255	56	525	724	261	111	Bkg	Bkg	909	3260	123	420	90	480
Lysine	510	Bkg	239	Bkg	Bkg	159	»	»	1716	4000	Bkg	Bkg	42	120
Histidine	Bkg	»	Bkg	»	»	Bkg	»	»	Bkg	Bkg	»	»	Bkg	Bkg
Arginine	»	»	119	»	»	»	»	»	»	246	»	»	»	»
Aspartic acid + Serine	2307	56	167	»	»	»	»	»	250	116	»	»	»	»
Glycine	510	1296	6333	67	362	270	»	»	2000	3882	175	574	70	120
Glutamic acid + Threonine	2290	920	167	Bkg	Bkg	85	111	297	486	116	Bkg	Bkg	21	200
Alpha-Alanine	922	Bkg	Bkg	»	90	Bkg	Bkg	212	Bkg	70	»	»		200
Tryptophane	Bkg	»	»	»	—	—	—	—	—	—	—	—	—	Bkg
Tyrosine	255	40	597	»	Bkg	Bkg	Bkg	—	122	70	90	Bkg	Bkg	»
Valine	1530	56	580	—	82	108	190	297	286	116	52	103	»	»
Leucine	357	380	580	153	186	274	190	85	254	309	385	383	84	880

Note: The radioactivity of amino acids (disint/min) is calculated per 1 g tissue (raw weight). Bkg = background.

some of the so-called "indispensable" amino acids in the animal organism. In our experiments the following amino acids were found to be radioactive:

Dispensable: Cystine (liver, muscles, intestines), aspartic acid (liver), glycine (liver, muscles, intestines), glutamic acid (liver, intestines), alpha - alanine (not always, liver, muscles, intestine), tyrosin (liver).

Indispensable: Lysine (liver, intestine), arginine (liver), valine (liver, intestine), leucine (liver, intestine).

The problem is undoubtedly of interest and is to be taken up in further investigations.

SUMMARY

Fishes are capable of absorbing carbon from mineral compounds disolved in water. Radioactive carbon, absorbed by fish from the surrounding media, appears in large quantities in blood serum, liver, bones, intestines, brain, scales, eyes and kidneys, and in lesser quantities in muscles, bile, and red blood cells. The inorganic carbon, having entered the fish organism, participates in the metabolism, and is converted from mineral into organic carbon-containing compounds, in particular into carbohydrates, proteins, and certain amino acids.

LITERATURE CITED

[1] T.V. Aristovskaya, Uspekhi Sovrem. Biol. 17, 1, 54 (1944).

[2] K. Werkman and P. Wilson, The Physiology of Bacteria [Russian translation] (IL, Moscow, 1954).

[3] S. Ruben and M. Kamen, Proc. Nat. Acad. Sci. 26, 418 (USA, 1944).

[4] E.A. Evans and Z. Slotin, J. Biol. Chem. 136, 301 (1940).

[5] A.K. Solomon, B. Vennesland, et al., J. Biol. Chem. 140, 171 (1941).

[6] A.M. Delluva and D.W. Wilson, J. Biol. Chem. 166, 739 (1946).

[7] C.B. Anfinsen, A. Beloff, etal., J. Biol. Chem. 168, 771 (1947).

[8] F.H. Armstrong, J. Shubert, and A. Lindenbaum, Proc. Soc. Exp.Biol. and Med. 68, 233 (1948).

[9] A.M. Brues and A. Waranjo, Anatom. Record 100, 644 (1948).

[10] A.S. Konikova, V.N. Orekhovich, and M.G. Kritsman, Doklady Akad. Nauk SSSR 65, 325 (1949).

[11] B.N. Stepanenko, G.N. Zubrilina and N.P. Khayurina, Doklady Akad. Nauk SSSR 100, 521 (1955).

[12] B.N. Stepanenko, Sessiya AN SSSR po mirn. isp. atomn. energii (1-5 July, 1955), (Izd. AN SSSR, Moscow, 1955).

[13] P.A. Marks and B.A. Khorekker, Mirn. isp. atomn. energii. Mezhdunar. konf. v Zheneve 12, 594 (1955).

[14] S. Karpiak, Wszechswiat 8, 188 (1956).

[15] O.A. Alekin, Hydrochemistry [in Russian] (Gidrometeoizdat, Leningrad, 1948).

[16] N.P. Meshkova and S.E. Severin, the Practice of Animal Biochemistry [in Russian] ("Sov. Nauka", Moscow, 1950).
[17] H. Bode, Biochem. Z. 326, 433 (1955).
[18] I.P. Ivanov, V.K. Modestov, Yu.M. Shtukkenberg, et al., Radioactive Isotopes in Medicine and Biology [in Russian] (Medgiz, Moscow, 1950).
[19] A.E. Gurvich, Lab. Delo No. 3 (1955).
[20] W.P. Jencks, M.R. Jetton, and E.Z. Durrum, Biochem. J. 60, 205 (1955).
[21] H.L. Tarr, Ann. Rev. Biochem. 27, 223 (1958).

ASCORBIC ACID METABOLISM IN THE WALLS OF THE AORTA IN DEVELOPMENT OF RADIATION INJURY

L.I. Polikarpova

Translated from Biokhimiya Vol. 25, No. 3, pp. 465-469,
May-June, 1960
Original article submitted August 21, 1959

Hemorrhage is one of the symptoms of radiation sickness. On the basis of literature data [1, 2, et al.] it can be assumed that one of the chief causes of the development of a hemorrhagic effect is the destruction of strength of the blood vessel walls.

At the present time there is experimental evidence that in hemorrhagic diathesis in the organism, especially in the vessel walls, there is destruction of the metabolism of the intercellular substance of connective tissue [3-5]. Ascorbic acid (AA) is of great value in preserving the stability of intercellular substance [3, 5]. In some cases under conditions of radiation injury there has been observed a considerable decrease in AA content in blood and tissues, especially in the adrenals, and also in muscles, kidney, liver, testes, stomach, small and large intestines. However, it should be remarked that the injection of AA in irradiated animals does not decrease the development of the hemorrhagic effect in them [6].

In order to study the mechanism of destruction of strength in the vessel walls in acute radiation sickness we carried out determination of the content of free AA and the product of its reversible oxidation, dehydroascorbic acid (DAA), in the tissues of the vessels of animals which synthesize AA (rabbits) and of those which do not (guinea pigs). As the object of study we used tissues of the aorta (removal of smaller vessels for biochemical study was not possible).

METHODS

The experiments were carried out on male rabbits weighting 2.0-2.5 kg and on male guinea pigs weighing 200-250 g. Up to the time of the experiment the animals were kept on the constant diet of the animal house, which for rabbits was hay, oats, and cabbage, and for guinea pigs, hay, oats, and carrots. Acute radiation sickness was produced by a single external irradiation with the γ-rays of Co^{60} (strength of dose 500 r/min) in a dose of 1000 r for rabbits and 800 r, guinea pigs. The rabbits were killed by air embolism, the guinea pigs by decapitation. For 16 hours before death the animals received no food. The aorta was removed from the body of the animal, and freed from surrounding tissues and blood; AA and DAA were determined in it by the method of Yarusova [11].

The concentration of AA and DAA in the aorta tissues was determined in rabbits 1, 3, 6, and 24 hours, 3 and 5 days after irradiation; in guinea pigs, after 1, 3, and 5 days. At the doses of ionizing radiation used, death of the animals occurred 5-7 days after the irradiation. Thus, the investigation within this period was carried out during the appearance of the acute form of radiation sickness.

For the determination of AA and DAA in each experiment we used 400 mg of aorta tissue, obtained from one rabbit or five guinea pigs, and we calculated the concentrations (in mg%) on net weight of tissue.

RESULTS

In Fig. 1 we give the experimental data which characterize the changes in total concentration of AA and DAA, and also these substances separately, in aorta tissues of rabbits during development of acute radiation sickness. We can see that even an hour after irradiation there develops a tendency to decrease in total amount of AA and DAA, and this follows from some decrease in content of both AA and DAA. Three hours after irradiation, both the total content of AA and DAA and the concentrations of AA and DAA separately had again reached the normal values. Later, beginning at six hours after irradiation, the total amount of AA and DAA showed a tendency to increase (with a maximum at 24 hours). However, this occured only because of a rise in concentration of AA, with a relatively stable level for the DAA content. During the rest of the time (3 and 5 days after irradiation there was a clear decrease in AA concentra-

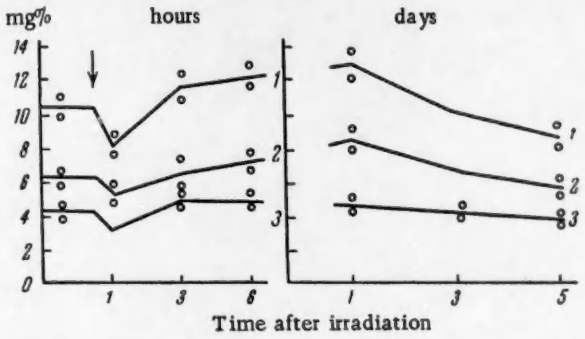


Fig. 1. Content of ascorbic acid (AA) and dehydroascorbic acid (DAA) (in mg%) in aorta tissues of rabbits in acute radiation Sickness 1) AA + DAA; 2) AA; 3) DAA. Dots show variation from average; arrow indicates moment of irradiation.

tion while the DAA concentration) during this time remained approximately at the normal level: The total content of AA and DAA fell.

For the guinea pigs we have given the results only at the end of the first day after the action of ionizing radiation. However, these results indicate a considerable difference in the separate reactions of the guinea pig as compared to the rabbit (Fig. 2).

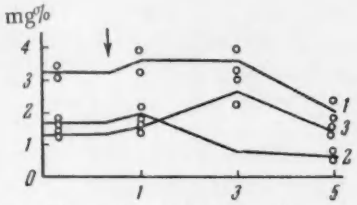


Fig. 2. Content of ascorbic acid (AA) and dehydroascorbic acid (DAA) (in mg%) in aorta tissues of guinea pig in acute radiation sickness Designations the same as in Fig. 1.

Under our conditions the total content of the AA and DAA in aorta tissues of the normal guinea pig was considerably less than in the rabbit. A day after the irradiation, both the total content of AA and DAA, and the AA and DAA separately, were maintained within normal limits in the guinea pig. On the third day after irradiation the total concentration of AA and DAA was also normal, but there was a sharp, contradictory change in the separate contents of AA and DAA: the amount of AA fell and the DAA rose. On the fifth day the total content of AA and DAA also fell, following upon the fall at this time of the DAA concentration, with small further decrease in AA concentration.

The very obvious difference in metabolic change for AA in the aorta tissues of rabbits and guinea pigs appears in the values for the ratio of amount of DAA and amount of AA (Table). This ratio, in particular, is evidence of the direction of oxidation-reduction processes in the tissues. The data given are averages, calculated on the basis of results of separate experiments, and not the ratio between average values for the content of DAA and AA.

These results show that the normal ratio of DAA/AA in each type of animal is approximately the same. A day after irradiation, in the rabbit there is a tendency for it to fall, and in the guinea pig it remains normal. On the third and fifth days the DAA/AA ratio rises to normal in the rabbit, while in the guinea pig it remains five times above normal on the average.

Since the guinea pig does not synthesize AA and the sole source in the organism is its accumulation from the diet, we must consider the possible role of the decreased appetite characteristic of radiation sickness, and the related undereating in the destruction of AA metabolism in the vessel tissues of these animals.

The amount of foodstuffs containing AA which was used by the guinea pigs during the first three days after irradiation was on the average the same as before irradiation, but on the fourth and fifth days this decreased. In this connection it was necessary to establish the significance of undereating for destruction of AA metabolism in guinea pig aorta tissue during this period.

We ran special experiments to determine the content of AA and DAA in guinea pig aorta tissue during five days on the same amount of food as for the irradiated animals. We showed that during this period the total amount of AA and DAA as a result of undereating was below normal, with a value of 2.3 ± 0.4 mg%, that is, at approximately the same level as in guinea pigs through a five-day period after irradiation, but in this case there was a difference in the separate changes of AA and DAA concentration. The AA content in undereating of the guinea pigs was 1.1 ± 0.3 mg%, that is, an average fall of 31% compared to the normal, while through five days after irradiation, it fell still more, by 61%. The amount of DAA in undereating averaged about the same as in the normal animals (1.2 ± 0.1 mg%).

DISCUSSION OF RESULTS

Our investigation showed that acute radiation sickness causes destruction of AA metabolism in the aorta tissue of animals which synthesize AA (rabbits) and those which do not synthesize it (guinea pigs). From this, we must consider two different directions in which the changes occur: 1) variation in total concentration of the components in the easily reversible process AA $\rightleftharpoons$ DAA + 2H; 2) shift in ratio DAA/AA which, as we showed, is characteristic for destruction of the oxidation-reduction process in the tissues. In rabbits, change begins even in the first day after the action of radiation. Immediately after irradiation there is a tendency in them for fall in the total amount of AA and DAA without

Ratio of Amounts of Dehydroascorbic Acid to Amounts of Ascorbic Acid (in %) in Aorta Tissues of Rabbits and Guinea Pigs in Acute Radiation Sickness

Species of animals	Normal	After irradiation					
		1 hour	3 hours	6 hours	1 day	3 days	5 days
Rabbit	67±5	62±2	80±6	70±7	52±3	68±5	80±7
Guinea pig	78±8	—	—	—	79±7	326±70	412±18

clear destruction of the oxidation-reduction potential; then there is a gradual rise in total amount of AA and DAA above normal which is accompanied by a shift to the side of the reduction process.

In the study of the later periods, we could compare the reaction of rabbits and guinea pigs. In rabbits, the total concentration of AA and DAA had a tendency to fall during five days after irradiation without destruction of the oxidation-reduction potential. In guinea pigs, the content of AA along with DAA also fell, but only from the third day, and without preliminary rise as occurred in rabbits. Then in the guinea pigs there was a sharp increase in oxidative processes which was not found in rabbits. If in rabbits and guinea pigs the normal content of AA was higher than of DAA, then while the same ratio was retained in radiation sickness in rabbits, it changed sharply toward the side of DAA in guinea pigs.

All these results indicate that the action of ionizing radiation causes a considerably greater qualitative change in AA metabolism in the aorta walls of guinea pigs than in rabbits.

Most of all we note the sharp deficit of free AA in guinea pigs on the third day after irradiation. This was conected only with the effect of the action of ionizing radiation, since during this period the amount of food required by the experimental animals was the same as for the normals. The AA deficit on the fifth day after irradiation certainly depended both on undereating and on irradiation of the organism.

Evidently, destruction of AA metabolism in radiation injury as seen in aorta tissue also occurs in the smaller vessels and is one of the factors which affect metabolism of intercellular substances in connective tissue and hence the permeability and fragility of the vessel walls.

It is important to observe that humans fall in the same group as guinea pigs with respect to AA metabolism. Probably, in the human blood vessel walls in acute radiation sickness analogous changes can occur.

SUMMARY

Acute radiation sickness causes changes in ascorbic acid metabolism in aorta walls in animals which synthesize ascorbic acid (rabbits) and in those which do not (guinea pigs).

In guinea pigs the content of ascorbic acid decreases 3-5 days after irradiation with a sharp increase in the amount of dehydroascorbic acid on the third day; in rabbits the content of ascorbic acid increases on the first day with further fall to normal in relation to the unchanged content of dehydroascorbic acid.

The ratio of the amount of dehydroascorbic acid to the amount of ascorbic acid (an indication of the direction of the oxidation-reduction processes in the organism) sharply increases in guinea pigs 3-5 days after irradiation, and in rabbits is unchanged.

In guinea pigs, the development of radiation sickness leads to a considerable deficit of ascorbic acid in the aorta walls, and in rabbits this is not found.

LITERATURE CITED

[1] N.A. Kurshakov, in: Biological Action of Radiation and Clinical Radiation Sickness [in Russian], (Medgiz, Moscow, 1954), p. 137.

[2] P.D. Gorizontov, in: Radiation Medicine [in Russian] (Medgiz, Moscow, 1955), p. 139.

[3] S.B. Wolbach, New England J. Med. 215, 1158 (1936).

[4] R. Chamber and B.W. Zweifach, J. Cellular and Compar. Physiol. 15, 255 (1940).

[5] A.H. Hunt, Brit. J. Surg. 28, 436 (1941).

[6] E.F. Romantsev and A.V. Savich, Chemical Defense Against Ionizing Radiation [in Russian] (Medgiz, Moscow, 1958), p. 122.

[7] K.S. Klimenko, Fiziol. Zhur. 3, 117 (1957).

[8] M.F. Merezhinskii, N.E. Glushakova, F.M. Laguto, and E.N. Yadvinskaya, Abstracts of Proceedings, XIII Scientific session, Inst. Nutrition AMN SSSR [in Russian] (Medgiz, Moscow, 1959), p. 69.

[9] C.H. Kretzschmar and F. Ellis, Brit. J. Radiol. 20, 94 (1947).

[10] H.L. Oster, A.L. Kretchmar, and F.H. Bethell, Proc. Soc. Exptl. Biol. and Med. 84, 470 (1953).

[11] N.S. Yarusova and V.S. Asatiani, Methods of Biochemical Investigation [in Russian] (Medgiz, Moscow, 1956), p. 361.

ADENYLIC ACID SYSTEM IN TISSUE CULTURE CELLS UNDER AEROBIC AND ANAEROBIC CONDITIONS

V.I. Agol and I.V. Skarlat

Biochemistry Laboratory, Poliomyelitis Research Institute,
Academy of Medical Sciences USSR, Moscow
Translated from Biokhimiya Vol. 25, No. 3, pp. 470-475,
May-June, 1960
Original article submitted August 22, 1959

In a number of investigations it was shown that tissue culture cells, independent of their source of origin (whether from normal or malignant tissues), possessed a characteristic metabolism, quite reminiscent of cancer-cell metabolism. One of the basic peculiarities of cells grown in vitro is their quite intense aerobic glycolysis* [1-7]. The presence of aerobic glycolysis, without doubt, reflects itself in a significant fashion on the tissue culture energy balance. In those cases when the cells are grown without oxygen, particular importance should be given to anaerobic glycolysis. The above data aroused the authors' interest to study the importance of glycolysis and respiration in supporitng the energy potential of tissue explant cells. The adenylic system components were chosen as the qualitative indicator characterizing the cellular energy state.

In the present work are presented data obtained during study of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) contents in cells of continuously-propagating heart culture of the monkey Macacus cynomolgous [9]; data were obtained under aerobic and anaerobic conditions and in the presence or absence of glucose.

METHODS

The growing of cells and taking them from the glass surface and washing was described in previous communication [6]. A definite aliquot of cellular suspension was fixed with cold trichloroacetic acid (TCA, final concentration 3.3%); simultaneously two similar samples were placed in special centrifuge containers containing 80 ml of Krebs-Ringer phosphate buffer (pH 7.3-7.4). One of the containers also contained glucose in concentration of 150 mg%. The incubation medium was preliminarily heated to 37°. The large incubation volume was chosen in order to minimize the influence of changes in substrate and end products concentrationson the cellular components of the adenylic system. In order to agitate the cells and create the corresponding gas medium, there was bubbled through the suspension air (aerobic conditions), or oxygen-free nitrogen (anaerobic conditions). The incubation interval was 20 min and temperature was 37°. A special check has shown that under the chosen conditions, glycolysis of tissue culture cells proceeded with uniform intensity. In addition, due to the large medium volume, its glucose concentration (Nelson [10]) remained basically unchanged during incubation, and lactic acid concentration (determined according to Baker and Summerson [11]) was relatively low. At the end of incubation the cells were centrifuged for three minutes and fixed with TCA.

The separation and quantitative determination of adenine nucleotides was conducted according to the ascending paper chromatography method. Prior to chromatography, TCA was removed from the extract by a five-fold ether extraction in cold. Control determinations have shown that with the method utilized (treatment with TCA and ether) there was no loss of adenine nucleotides, nor breakup of ATP. The volume of the extract transferred onto the paper (this was preliminarily washed with 0.5% EDTA and the solvent) was 0.1-0.2 ml. In order to diminish the size of the spot, the extract was transferred in small portions and constantly dried in an air stream. A mixture of isoamyl alcohol–2.5% citrate was utilized as the solvent [12]. In order to improve the nucleotide separation, each sample was generally chromatographed three times. The spots were developed with the help of an ultrachemiscope. Full elution was accomplished by eluting with 0.01N HCl in a shaking apparatus for one hour. Small paper hairs were removed from the elution by rapid centrifugation (at about 8000 rpm). Concentration of adenylic compounds in the eluent was determined by photometry at 260 mμ inan SF-4 spectrophotometer. Molar extinction for ATP, ADP and AMP was taken as 14,200 In some cases there was determined the ratio between nucleotide adenine and phosphorus; phosphorus concentration in the elutant was determined by the color reaction with molybdenum in the presence of ascorbic acid [13]. The obtained values agreed satisfactorily with theoretical expectations.

All calculations were conducted in terms of 1 ml of solid cell precipitate. Cell concentration in the sus-

* A recent attempt to prove that under physiological conditions tissue culture cells do not undergo aerobic glycolysis [8] cannot be accepted as sufficiently convincing. Numerous facts, all of which cannot be analyzed presently, attest to the fact that aerobic glycolysis is present in multiplying, biologically complete tissue explants.

pensions was determined with a rapid hematocrit centrifuge in capillaries 75 mm long. A check showed that 1 ml of cellular precipitate contained 170-180 million cells (calculations were conducted on crystal violet – stained nuclei after disrupting the cytoplasm with citric acid).

Statistical treatment of the obtained material was conducted by generally accepted methods [14].

RESULTS

Adenylic acid system in initial cells. Determination of the total content of adenine nucleotides (in μ moles/ml of solid cellular precipitate) and their distribution according to fractions gave the following results:

Total adenine nucleotide content

3.75 ± 0.18 (13)

ATP, %	ADP, %	AMP, %
37.8 ± 2.27	43.1 ± 2.47	19.1 ± 1.19

Note: Here and in the following tables are given arithmetic means and their standard errors. In parentheses-number of determinations.

The low relative ATP concentration and the high ADP concentration in these cells deserve definite attention. Consequently, the cells contain a considerable store of phosphorus-acceptors necessary for oxidative, as well as for glycolytic, phosphorylation. It should not be forgotten, however, that at the time of removal of the cells from glass and their washing, there could take place a partial breakup of ATP. Thus, it was quite possible that in growing cells there could be a somewhat different adenine nucleotide distribution - more ATP and less ADP. Together with this, peculiarities in cells removed from glass and washed present a separate interest, namely since such cells are utilized for manometric experiments.

The influence of incubation conditions on total content of adenine nucleotides in tissues culture cells. Data concerning the influence of aerobiosis and anaerobiosis on adenine nucleotide content of tissue culture cells, incubated in the presence and absence of glucose, are shown in Table 1. Results of statistical treatment of these data are given in Table 2.

It is not difficult to see that the total content of components of the adenylic acid system in cells incubated under aerobic conditions was not significantly dependent on the presence of glucose. In other words, endogenous respiration was sufficient for maintenance of the level of cellular adenine nucletides. On the other hand, glycolysis alone (as shown by data from anaerobic experiments conducted in the presence of glucose) was likewise capable of maintaining total adenine nucleotide content at a steady level. But in the absence of respiration and glycolysis (anaerobic experiments without glucose) the reserves of adenylic acid coenzymes were rapidly exhausted. This occurrence could depend on fuller disintegration of adenylic acid system components, or could be due to their escape from the cells as a result of increased permeability.

TABLE 1. The Influence of Incubation Conditions on Total Content of Adenine Nucleotides in Tissue Culture Cells. Nucleotide Content Expressed in μmoles per ml of Solid Precipitate.

Aerobiosis		Aerobiosis	
with glucose	without glucose	with glucose	without glucose
3.45 ± 0.41 (6)	3.05 ± 0.49 (4)	3.07 ± 0.21 (6)	1.84 ± 0.13 (5)

Influence of incubation conditions on the ATP, ADP and AMP content in tissue culture cells. The dependence of adenylic acid system distribution, in separate fractions, on the composition of the gaseous medium and on the presence or absence of substrate is presented in Table 3. Results of statistical treatment of these data are presented in Table 4.

The obtained data attest to the fact that under aerobic conditions, the presence or absence of glucose was not basically reflected in the cellular ATP content. This speaks of the fact that endogenic respiration was sufficiently effective in the sense of supporting cellular energy potential at a definite level. Together with this, it should be noted that in the absence of glucose there was some lowering of the ADP portion and increase in AMP in the total sum of adenine nucleotides.

TABLE 2. Significance of Differences (p) in Adenine Nucleotide Content of Tissue Culture Cells Under Different Conditions.

Incubation conditions	Aerobiosis	Anaerobiosis	
	Without glucose	with glucose	without glucose
Aerobiosis with glucose	0.6 > p > 0.5	0.5 > p > 0.4	0.01 > p > 0.001
Aerobiosis without glucose	–	p > 0.9	0.05 > p > 0.02
Anaerobiosis with glucose	–	–	0.001 > p

TABLE 3. Influence of Incubation Conditions on the Distribution of Adenine Nucleotides in Tissue Culture Cells According to Individual Fractions.

Adenylic acid system fractions	Aerobiosis		Anaerobiosis	
	with glucose	without glucose	with glucose	without glucose
ATP	38.0 ± 2.14	40.0 ± 3.87	40.7 ± 1.83	32.4 ± 1.50
ADP	43.0 ± 2.13	33.4 ± 3.11	42.7 ± 1.47	30.3 ± 2.97
AMP	19.0 ± 0.92	26.6 ± 1.69	16.6 ± 2.10	37.3 ± 2.20

TABLE 4. Significance of the Differences in the Individual Nucleotide Content in Tissue Culture Cells, Incubated in the Presence and Absence of Glucose.

Adenylic acid system fractions	Aerobiosis	Anaerobiosis
ATP	$0.7 > p > 0.6$	$0.01 > p > 0.001$
ADP	$0.05 > p > 0.02$	$0.01 > p > 0.001$
AMP	$0.01 > p > 0.001$	$0.001 > p$

Another important condition was the fact that in the presence of glucose the adenylic acid coenzyme content was independent of the gas medium composition; the ATP, ADP and AMP content in cells incubated under anaerobic conditions was practically the same as the content of these nucleotides in cells incubated in the presence of oxygen. Consequently, tissue-culture cells can effectively resynthesize ATP at the expense of anaerobic glycolysis.

The most drastic changes, as could be expected, occur in cells incubated under anaerobic conditions without glucose. Under these conditions there is a considerable decrease in the ATP and ADP portions and a concurrent increase in AMP. If it is taken into account that the total adenine nucleotide content under these conditions was also considerably lowered, then the depletion of neuroenergetic compounds from cells becomes even more evident.

DISCUSSION

The importance of respiratory and glycolytic energy to tissue culture cells. The obtained data shows that monkey heart tissue-culture cells were able to support their energy potential (at least in short-term experiments) at the expense of endogenous respiration as well as at the expense of glycolysis. Taking into account the similarity of cellular carbohydrate and oxidative metabolisms in different tissue cultures [6], it could be supposed that the presently observed peculiarities in energy metabolism are a general characteristic of various tissue explants. In this respect, tissue-culture cells are reminiscent of cancer cells [15-17], leucocytes [18] and some other cells.

However, to what degree the ATP was formed in the cytoplasm during glycolysis, biologically equal to mitochondreal ATP formed during respiration, is yet somewhat difficult to say [20]. It seemed that the answer to this question could be found in the results of the study concerning the ability of tissue-culture cells to grow under anaerobic conditions. However, first of all, the literature concerning this question is quite controversial: at the same time that some authors state that tissue explants multiply normally in the absence of oxygen [21 - 23], others refute this possibility [24, 25]. Secondly, and this is especially significant, even if it was concretely proven that tissue cultures do not multiply under anaerobic conditions, it would not to any degree exclude the aspect of the energy value of glycolysis. Absence of multiplication could be dependent, for example, on the absence of substrates that are formed only during oxidative processes. In order to solve the question concerning the energy value of glycolysis, emphasis should be placed upon experiments concerning the study of intracellular transfer of adenylic system coenzymes between mitochondria and cytoplasm. If such a transfer is easily accomplished, then evidently, from the energy point of view, ATP formed during glycolysis should be of the same value as ATP formed during the respiratory process.

Effectiveness of oxidative phosphorylation in the cell. The effectiveness of oxidative phosphorylation, i.e., concerning the quantity of ATP formed during utilization of a definite amount of oxygen, is generally judged by experiments with isolated cellular fractions. Whole cells present such a complex system that obtaining of a somewhat reliable analysis of oxidative phosphorylation effectiveness becomes somewhat unaccomplishable. However, lately Quastel and Bickis [26] have suggested a method for determining the ratio P:O for whole cells possessing a reverse Pasteur reaction.

The basis of the method is based on the following. If addition of glucose under aerobic conditions promotes a supression of oxygen utilization and appearance of

aerobic glycolysis, but does not produce basic changes in ATP content, then it can be assumed that formation of energy during glycolysis (1 mole of ATP per mole of lactate formed) fully compensated the loss of energy related to the lowered respiration (allowing that utilization of ATP for plasticity and other goals did not depend on the presence of glucose). From this it is easy to calculate the ratio P:O by the following equation:

$$(Q_{O_2} - Q^{gl}_{O_2}) \cdot 2\frac{P}{O} = Q^{O_2}_{CO_2},$$

where Q_{O_2} and $Q^{gl}_{O_2}$ are utilization of oxygen in the absence and presence of glucose respectively, and $Q^{O_2}_{CO_2}$ represents aerobic glycolysis.

Since addition of glucose to tissue-culture cells from monkey heart produced a lowering in oxygen utilization and the appearance of aerobic glycolysis [6] and since in the present publication it was shown that during this there did not occur any basic changes in the cellular ATP concentration, evidently, then, the method of Quastel and Bickis can be utilized for the present objectives. Corresponding calculation, based on previously obtained data [6], shows that for whole monkey heart culture cells the ratio P:O was about two. It can be pointed out that for cells from Ehrlich's ascites-carcinoma, Quastel and Bickis obtained P:O = 3.0, and other authors, 2.5 [27]. Without reevaluating the accuracty of the obtained figures, a conclusion should be made that respiration in tissue-culture cells was accomplished, evidently, approximately with the same effectiveness (with respect to formation of macroenergy bonds) as in other normal and malignant tissues.

Concerning the mechanism of reverse Pasteur effect. The reverse Pasteur reaction in tissue culture cells was not accompanied by a basic lowering of the level of phosphate acceptors (ADP); this fact seemed important. More readily, in the opposite, the addition of glucose under aerobic conditions assisted the increase in ADP concentration at the expense of AMP. This condition could also speak out against propositions stating that exhaustion of phosphate acceptors as a result of competition for ADP by glycolysis and respiration, formed the basic for the reverse Pasteur reaction [28]. However, the final conclusion concerning this relation will not be made until after the study of intracellular distribution of adenine nucleotides, since the respiratory suppression could be accomplished by a local insufficiency of mitochondrial phosphorus-acceptors.

SUMMARY

The adenine nucleotide content in heart tissue-culture cells from the monkey, Macacus cynomolgus, incubated under aerobic and anaerobic conditions in the presence and absence of glucose, was studied by the method of paper chromatography. It was shown that the cellular ATP level could be supported at the expense of endogenous respiration as well as by anaerobic glycolysis. Addition of glucose to cells under aerobic conditions was accompanied by a slight increase in ADP concentration (at the expense of AMP). The obtained results are discussed in relation to the problem of the effectiveness of oxidative phosphorylation in the whole cell in connection with the mechanism of reverse Pasteur effect.

LITERATURE CITED

[1] A.A. Krontovskii and I.A. Bronshtein, Zh. exp. biol. i med. 2, 1 (1926).
[2] O. Warburg, Naturwiss. 46, 25 (1959).
[3] M. Green, G. Henle and F. Deinhardt, Virology 5, 206 (1958).
[4] B.B. Westfall V.J. Evans, et al., Cancer Res. 18, 947 (1958).
[5] V.H. Gershanovitch, V.I. Agol, R.N. Etingoff and S.G. Dzagurov, Biokhimiya 23, 453 (1958).†
[6] V.I. Agol, V.H. Gershanovitch and R.N. Etingoff, Biokhimiya, 24, 101 (1959).
[7] R.H. Etinoff, M.N. Khanina and V.N. Gershanovitch, Vopr. Med. Khimii 5, 29 (1959).
[8] T. Luthardt, T. Sauthoff, and H.K. Mittelstrass, Z. Naturforsch. 14, 253 (1959).
[9] J.E. Salk, Am. J. Public Health 47, 1 (1957).
[10] N.A. Nelson, J. Biol. Chem. 153, 375 (1944).
[11] S.B. Barker and W.H. Summerson, J. Biol. Chem. 138, 535 (1941).
[12] I.A. Sytinskii, Biokhimiya 21, 359 (1956).†
[13] T.Y. Venkstern and A.A. Baev, Biokhimiya 22, 1043 (1957).†
[14] M.I. Mostkovyi, Practice in Variance Analysis of Clinical Material. [in Russian] (Izd Pub. AN Turkmen SSR, Ashkhabad, 1954).
[15] W. Kunz and W. Schmid, Z. Naturforsch 12, 743 (1957).
[16] K. Ibsen, E.L. Coe, and R.W. McKee, Biochim. et biophys. acta 30, 384 (1958).
[17] M.F. Siyanitzkaya and I.F. Seitz, Collection: Actual Questions in Blood Transfusion 6 [in Russian] (Institute of Blood Transfusion, Leningrad, 1958), p. 189.
[18] I.S. Luganova, I.F. Seitz and V.I. Teodorovitch, Vopr. Med. Khimii 3, 428 (1957).
[19] V.I. Agol, Uspekhi Sovrem. Biol. 49, 37 (1960).
[20] O. Warburg, Science 123, 309 (1956).
[21] H. Laser, Biochem. Z. 264, 72 (1933).
[22] H. Harris, Brit. J. Exptl. Pathol. 37, 512 (1956).
[23] M. Jones and S.L. Bonting, Exptl. Cell. Res. 10, 631 (1956).
[24] D.M. Pace and H.J. Phillips, Proc. Soc. Exptl. Biol. and Med. 90, 568 (1955).
[25] F. Windisch and H. Haehn, et al., Naturw. 45, 287 (1958).
[26] J.H. Quastel and I.J. Bickis, Nature 183, 281 (1959).
[27] K. Ibsen, E.L. Coe, and R.W. McKee, Nature 183, 1471 (1959).
[28] B. Chance and B. Hess, Ann. N.Y. Acad. Sci. 13, 1008 (1956).

†Original Russian pagination. See C.B. Translation.

THE ASSIMILATION OF LABELED AMMONIA FROM THE SOIL BY THE ROOT SYSTEM

V.L. Kretovich, Z.G. Evstigneeva, and K.B. Aseeva

A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 476-481,
May-June, 1960
Original article submitted August 24, 1959

The study of biochemical processes underlying the incorporation by plants of inorganic nitrogen (N_{inorg}) and its conversion into organic forms—amino acids, amides, and proteins—is of enormous theoretical and practical value since only plant organisms are able to synthesize unsubstituted amino acids and complete proteins from inorganic compounds.

In his time Sabinin [1] advanced and experimentally developed the hypothesis that the root system should be regarded not only as an absorptive organ but also as an organ of synthesis of a number of the most important organic compounds. This hypothesis received experimental support in the studies of Shmuk et al [2], who demonstrated the leading role of the root system in the biosynthesis of alkaloids in tobacco. In recent years these views have been developed further in the studies of Kursanov [3], Mothes [4], Il'in [5], Potapov [6], Yemm [7], and others.

One of the methods by which the synthetic activity of the root system may be studied is to examine the sap composition. An examination of the forms of nitrogen in the sap is of great interest in the understanding of processes of assimilation of N_{inorg} entering the plant from the soil. It must be emphasized that in order to arrive at a correct view of the paths of N_{inorg} incorporation it is especially important to study sap composition of plants grown in their natural environment, i.e, in the soil. As our recent studies of the nitrogen composition of sap of pumpkins grown on soil containing both ammonium and nitrate fertilizers showed, there are present, together with nitrates, which constitute the greater part of the nitrogen, a whole series of amino acids, amides, and proteins [8].

The purpose of this study was the identification of those organic nitrogen compounds which represent the primary products of N_{inorg} assimilation and which are subsequently transported to the aerial parts of the plant.

METHODS

Experiments were performed with the sap of Gribov 31 pumpkin hybrids in the summer of 1957 and 1958. Pumpkins were grown in soil-containing flats in a growing house. The soil consisted of a mixture of humus, peat, and sand. During the month-long growing period, organic fertilizers were applied twice. At the time of sap collection the plants were well developed and had a vigorous root system. Before the beginning of sap collection, one hour before stem sectioning, NH_4NO_3 was applied to the soil as a 0.1N aqueous solution. The NH_4NO_3 preparation used in 1957 contained the stable isotope N^{15} and was enriched 30-fold, or 11 atom %. The preparation used in 1958 was enriched 25-fold, or 9.5 atom %. Five liters of a 0.14% solution of $N^{15}H_4NO_3$ solution was applied to each flat containing six to seven plants. This was done three times daily.

Sap was collected in the following manner: A collar of filter paper was fixed around the stem to prevent soil particles from falling on the stem and into the sap, and the stem was carefully scrubbed with alcohol. The stem was then cut off with a razor 2-3 cm above the root crown and the cut surface was washed with alcohol; a carefully washed and sterilized rubber pacifier was applied to the cut surface by the narrow end, through which a small hole had been made previously. The upper, wider end was covered with sterile cotton. As the sap accumulated, it was collected with sterile pipettes and stored at 0°. Sap was collected for 24 hours. Sap collected during the first nine hours was pooled and studied, and that collected during the next 15 hours was also pooled and studied. Data on amounts of sap collected are presented in Table 1.

The following determinations were made: total nitrogen (N_{tot} by the Kjeldahl method, N_{tot} after reduction with Zn according to Ballentain [8]; protein

TABLE 1. Quantities of Sap Collected. First and second growing periods.

Indicator	April-May		May-June		July-August	
	1957	1958	1957	1958	1957	1958
No. of plants	175	160	175	182	151	164
	First collection					
Quantity of sap in ml	647	1500	840	850	600	675
	Second collection					
	425	500	1100	1100	650	850

nitrogen (N_{prot}) after precipitation with tannin; ammonia nitrogen (NH_3-N) and amide N of asparagine and glutamine after removal of protein (amide N separately for the two amides [9]). For further analysis of the N^{15} content, samples obtained after determination of N_{tot}, N_{prot}, NH_3-N, and amide N, which contained nitrogen in the form of $(NH_4)_2SO_4$, were acidified with a few drops of 0.02N H_2SO_4, evaporated to a volume of 3 ml, and converted to gaseous nitrogen by the method of Rittenberg [10]. The nitrogen gas obtained was then analyzed in an MI-13-01 mass spectrometer for N^{15} content. Determination were made in triplicate or quadriplicate. All necessary controls were included.

As a sample of calculations of amide nitrogen of asparagine and glutamine, and of N^{15} enrichment of these fractions, made from data of May-June, 1957, collection I, we have:

Forms of nitrogen	N content of 5 ml of sap, mg	N^{15} enrichment factor
NH_3-N	0.064	11.97
Amide N of glutamine + NH_3-N	0.144	10.86
Amide N of asparagine and glutamine + NH_3-N	0.195	10.18

From these data we find that amide N of glutamine comprised 0.080 mg and amide N of asparagine 0.052 mg. The enrichment factor of the amide N of glutamine (X_1) was calculated from the equation:

$$a = b + c \qquad (I)$$

where a is the enrichment factor of the amide N of glutamine plus NH_3-N multipled by the amount of N in mg obtained in the determinations; b is the amount of NH_3-N in mg multiplied by its enrichment factor, c is the amount of amide N of glutamine in mg multiplied by its enrichment factor (X_1). The expression for calculation of the enrichment factor of the amide N of asparagine (X_2) has the form:

$$a_1 = b_1 + c_1 + d_1 \qquad (II)$$

where a_1 is the enrichment factor of the amide N of asparagine and glutamine and of NH_3-N multiplied by the amount of N in mg experimentally determined; b_1 is the amount of amide N of asparagine in mg multiplied by its enrichment factor (X_2); c_1 is the amount of NH_3-N in mg multiplied by its enrichment factor; and d_1 is the amount of amide N of glutamine in mg multiplied by its enrichment factor (X_1). Substituting the proper values into equations I and II, we have for glutamine: $10.86 \cdot 0.144 = 0.064 \cdot 11.97 + 0.080 \cdot X_1$ from which X_1 (enrichment factor) = 9.97; for asparagine we have: $10.18 \cdot 0.195 = 0.052 \cdot X_2 + 0.064 \cdot 11.97 + 0.080 \cdot 9.97$, from which X_2 (enrichment factor) = 8.09.

RESULTS

From the data presented in Table 2 it can be seen that sap of pumpkins grown in soil contains, in addition to very large amounts of nitrates as determined by zinc degradation, amide N of asparagine and glutamine and a little protein N. In the majority of experiments the quantity of NH_3-N is quite considerable. It should be noted that the sum of protein N, NH_3-N, and amide N sometimes exceeds N_{tot}, as determined by the Kjeldahl method; this is explained by the effect of nitrates and tannin added to the sap during precipitation of proteins and determination of amides and NH_3-N. The special features of determination of form of nitrogen in sap are considered in greater detail in a previous communication [8].

From a comparison of protein N contents of sap from the first and second collections it is evident that of the first collection is always somewhat higher by about 30-36%. With respect to amide N, the amount of this fraction is always lower in sap of the second collection; this is evidently related to a deficiency of carbohydrates in the roots as a result of cessation of flow of photosynthates into the root system. This also explains the fact that the amount of NH_3-N in the second collection is considerably higher, being on the average 142% of that in the first collection. The level of N^{15} of all fractions was determined. Data obtained are presented in Tables 3-4.

TABLE 2. Forms of Nitrogen in Pumpkin Sap

Forms of nitrogen	April-May				May-June				July-August			
	1957		1958		1957		1958		1957		1958	
	1st coll.	2nd coll.	1st coll.	2nd coll.	1st coll.	2nd coll.	1st coll.	2nd coll.	1st coll.	2nd coll.	1st coll.	2nd corr.
N_{total} after reduction with Zn	—	—	1,324	1,428	—	—	1,781	1,081	—	—	2,494	2,114
N_{total} by Kjeldahl	0,434	0,399	0,280	0,110	0,458	0,951	0,326	0,125	1,720	1,378	0,426	0,259
N_{prot}	0,050	0,068	0,025	0,036	0,088	0,055	0,038	0,044	0,038	0,039	0,030	0,032
NH_3-N	0,250	0,286	0,258	0,368	0,064	0,069	0,172	0,268	0,342	0,397	0,214	0,328
Amide N of glutamine	0,181	0,064	0,090	0,014	0,080	0,120	0,027	0,044	0,369	0,264	0,133	0,055
Amide N of asparagine	0,090	0,046	0,113	0,035	0,052	0,024	0,094	0,023	0,230	0,172	0,071	0,025

TABLE 3. Content of N^{15} in Various Nitrogen Fractions of Pumpkin Sap (Experiments of 1957)

Form of nitrogen	April-May						May-June						July-August					
	1st coll.			2nd coll.			1st coll.			2nd coll.			1st coll.			2nd coll.		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
N_{tot} after reduction with Zn	4,45	11,73	4,07	4,56	12,00	4,18	1,41	3,81	1,03	2,15	5,65	1,77	1,65	4,35	1,27	2,19	5,78	1,81
N_{tot} by Kjeldahl	1,06	2,81	0,68	1,74	4,59	1,36	0,56	1,47	0,18	0,71	1,86	0,33	0,74	1,97	0,36	0,98	2,59	0,60
N_{prot}	6,76	17,80	6,38	7,56	19,90	7,18	4,55	11,97	4,17	4,82	12,68	4,44	3,17	8,35	2,79	3,53	9,37	3,15
Amide N of glutamine	—	—	—	—	—	—	3,78	9,97	3,40	3,24	8,53	2,86	1,98	5,23	1,60	2,56	6,74	2,18
Amide N of asparagine	—	—	—	—	—	—	3,07	8,09	2,69	2,04	5,37	1,66	2,23	5,87	1,85	2,75	7,26	2,37

TABLE 4. Content of N^{15} in Various Nitrogen Fractions of Pumpkin Sap (Experiments of 1958)

Form of nitrogen	April-May						May-June						July-August					
	1st coll.			2nd coll.			1st coll.			2nd coll.			1st coll.			2nd coll.		
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
N_{tot} by Kjeldahl	4,87	12,81	4,49	5,54	14,58	5,16	3,48	9,15	3,10	4,13	12,18	3,75	4,28	11,26	3,90	4,85	12,76	4,48
N_{tot} after reduction with Zn	1,75	4,60	1,37	1,75	4,60	1,37	1,36	3,57	0,96	1,55	4,08	1,17	1,38	3,63	1,00	1,35	3,55	0,97
N_{prot}	1,16	3,05	0,78	1,37	3,60	1,00	1,05	2,23	0,67	1,69	4,45	1,31	0,96	2,52	0,58	1,82	4,78	1,44
NH_3-N	7,05	18,55	6,67	6,80	17,89	6,42	7,40	19,47	7,02	7,15	18,82	6,77	7,45	19,60	7,07	7,50	19,74	7,12
Amide N of glutamine	9,05	23,81	8,67	8,67	22,82	8,29	4,54	11,94	4,16	8,28	21,79	7,90	—	—	—	7,75	20,40	7,37
Amide N of asparagine	5,46	14,36	5,08	2,86	7,52	2,48	6,37	16,75	6,00	3,76	9,89	3,38	—	—	—	3,64	9,57	3,26

It is clear from the data that the ammonium salts in the sap had a high level of N^{15} enrichment. It is also shown that ammonium salts introduced into the soil are rapidly assimilated by the root system and enter the sap for further transport to the aerial parts of the plant. At the same time, the data bear abundant witness to the fact that nitrogen of ammonium salts entering the root system is already partially converted into organic form in the roots. NH_3-N is incorporated into the amide groups of asparagine and glutamine with especial ease. This is evident from the fact that the level of enrichment of the amide groups is extremely high. This is in excellent agreement with the data of Kretovich, Evstigneeva, and Plyshevskaya [11] showing that with introduction of N^{15}-labeled ammonium salts into lupine and vetch seedlings and also into sugar beet roots there is a particularly rapid incorporation of heavy nitrogen into the amide groups of asparagine and glutamine.

The lowest enrichment was observed in the fraction determined according to Ballentain with zinc and in the protein nitrogen fraction. The low enrichment level of the total nitrogen fraction determined with zinc is due to a dilution by the unlabeled nitrogen of nitrates present in very large quantities in sap of pumpkins grown in soil [8]. As far as the low enrichment of protein nitrogen is concerned, this indicates that although protein synthesis occurs in the root system it is relatively slow.

An examination of the data on enrichment of the various nitrogen fractions reveals that in all the experiments total nitrogen as determined by the Kjeldahl method (ammonia, amino acids, amides, and protein) and protein nitrogen of the second collection are more enriched with heavy nitrogen than the same fractions from the first collection. These data are in good agreement with the results of quantitative determinations of forms of nitrogen presented in Table 2. They denote that with the cessation of flow of carbohydrates and keto acids to the root system there is a more vigorous incorporation of heavy nitrogen into proteins and other compounds the nitrogen of which is determined by the Kjeldahl method.

From our data it is evident that processes of synthesis of organic nitrogen compounds in the root are most closely involved with photosynthesis and the flow to the root system from the leaves of non-nitrogenous carbohydrates [12].

DISCUSSION

The data obtained in the course of a study of incorporation of labeled nitrogen of ammonium into various nitrogen compounds of the sap clearly show that the greatest incorporation is into the amide groups of asparagine and glutamine. These two compounds, together with amino acids formed by direct amination of keto acids, are therefore the forms in which inorganic nitrogen from the soil is assimilated and stored by the root system. Here it is appropriate to recall that Bollard [13], who studied the nitrogen compounds of the xylem sap of a considerable number of plants belonging to widely separated families, showed that in almost all the plants investigated glutamine and asparagine were quantitatively the most important compounds present.

Kretovich and Uspenskaya [14], and also Kretovich and Yakovleva [15], showed that with introduction of ammonium salts into pea and wheat seedlings, and also into ripening wheat heads, there is a vigorous synthesis of glutamine. They also showed that introduction of keto acids causes a rapid disappearance of glutamine, which is evidently utilized in transamination reactions. A similar utilization of asparagine in the presence of oxalacetic acid was also shown for extracts of wheat and barley seedlings by Kretovich and Galyas [16].

It is apparent, therefore, that asparagine and especially glutamine are most important compounds, representing the immediate products of assimilation of ammonium nitrogen and its conversion into organic form by direct amination of keto acids and unsaturated acids.

The results of in vivo experiments with labeled nitrogen which are reported here are incontrovertible proof of the synthetic activity of the root system. Moreover, these results, obtained with new material and with the isotope method, develop more fully the profound conception of Pryanishnikov of the preminent role of amides in the conversion of exogenous ammonia from the soil into organic nitrogen compounds.

SUMMARY

Ammonium assimilation by the pumpkin root system was investigated by means of sap analysis. The pumpkin plants were grown in a soil to which ammonium nitrate labeled with N^{15} in the ammonium group was added.

Labeled ammonium ions were shown to be rapidly assimilated by the root system and to enter green parts of plants with the sap.

A part of the ammonium nitrogen undergoes charges in the root system itself and enters into the organic nitrogeneous compounds of the sap. Ammonium nitrogen enters into the amide groups of asparagine and glutamine at a particularly high rate.

The data obtained show that the nitrogen of exogenous ammonium salts is also incorporated into protein of the sap. This process, however, is relatively slow.

The results concerning the content of various nitrogen forms in sap and their enrichment with heavy nitrogen bear witness that synthesis of nitrogenous substances in the root system is closely associated both with photosynthesis in green parts of the plant and with translocation of nitrogen-free carbon compounds from the leaves into the root system.

LITERATURE CITED

[1] D.A. Sabinin, Timiryazev Lectures, Vol. IX [in Russian] (Izd. AN SSSR, Moscow, 1949).

[2] A.A. Shmuk, A.I. Smirnov, and G.S. Il'in, Doklady Akad. Nauk SSSR 32, 365 (1941).
[3] A.L. Kursanov. Vopr. Botan. 1, 131 (1954); Izvest. Akad. Nauk SSSR, Ser. Biol. 6, 689 (1957).
[4] K. Mothes, Wiss. Ann. Dtsch. Akad. Wiss. 5, 1 (1956).
[5] G.S. Il'in, Doklady Akad. Nauk SSSR 59, 1325 (1948); Wiss. Berlin Kl. Chem. 7, 113 (1956).
[6] N.G. Potapov and E. Cseh, Acta bot. Acad. scien. hung. 11, 147 (1955).
[7] J.W. Yemm and B.F. Folkes, New Phytologist 57, 106 (1958).
[8] V.L. Kretovich, Z.G. Evstigneeva, et al., Fiziol. Rastenii 6, 18 (1950).
[9] V.L. Kretovich and Z.G. Evstigneeva, Doklady Akad. Nauk SSSR 66, 429 (1949).
[10] D. Rittenberg, The Isolation and Determination of Labeled Atoms. Collected Papers [Russian translation] (IL, Moscow, 1948).
[11] V.L. Kretovich et al., Doklady Akad. Nauk SSSR 109, 1001 (1956).
[12] A.L. Kursanov, Timiryazev Lectures, [in Russian] (Izd. AN SSSR Moscow 1959) (in press).
[13] E.G. Bollard, Austr. J. Biol. Sci. 10 (3), 292 (1957).
[14] V.L. Kretovich and Zh. V. Uspenskaya, Biokhimiya 24, 116 (1959).*
[15] V.L. Kretovich and V.I. Yakovleva, Fiziol. Rastenii 2, 165 (1959); Doklady Akad. Nauk SSSR 125, 210 (1959).
[16] V.L. Kretovich and E. Galyas, Doklady Akad. Nauk SSSR 124, 217 (1959).

*Original Russian pagination. See C.B. Translation.

THE CYTOCHROME SYSTEM IN THE CYTOPLASMIC MEMBRANES OF Micrococcus lysodeikticus

N.S. Gel'man, M.A. Lukoyanova, and A.I. Oparin

A.N. Bakh Institute of Biochemistry,
Academy of Sciences USSR, Moscow
Translated from Biokhimiya Vol. 25, No. 3, pp. 482-486,
May-June, 1960
Original article submitted August 25, 1959

It is now established that oxidation–reduction enzyme systems are localized in structural elements of the cell and require for effective operation the maintenance of a definite spatial organization.

We have previously shown that oxidation of succinic acid by a system of enzymes which are subsumed under the term succinoxidase takes place in intact Micrococcus lysodeikticus cells and also in protoplasts obtained by lysis in osmotically stabilized medium [1, 2]. In a cell lysate prepared in weak salt solution, the oxygen absorption obtained with succinic acid as a substrate is only 10% that of intact cells. This residual absorption is entirely associated with the cytoplasmic membranes, which are preserved during lysis in osmotically unstabilized medium [3]. Although the succinoxidase activity of these structures is not large, they contain up to 70% of the succinic dehydrogenase of the original lysate and are characterized by a high specific activity of this enzyme [2]. The localization of succinic dehydrogenase in the cytoplasmic membranes indicates that the complete succinoxidase system in the intact cell is concentrated in these structures. The decrease in succinic acid oxidation with lysis is probably due to a change in the spatial organization of other components of the complex succinoxidase system.

The purpose of this study is to identify the components of the succinoxidase system in the cytoplasmic membranes and to clarify their relation to succinic dehydrogenase.

METHODS

The Fleming strain of a Micrococcus lysodeikticus culture served as experimental material. Culture and harvesting of the bacteria were carried out according to a previously described method [4]. Lysozyme and cytochrome c were obtained from the Light firm, and DNAase from the Lachem firm. Lysis and isolation of the cell membranes from the lysate were carried out as follows: Two g of bacteria (moisture content 70-75%) were suspended in 12.5 ml of 0.001M K-Na phosphate buffer, pH 6.8, containing 2.5 mg $MgSO_4$ per ml. This buffer was used in all succeeding operations. Five mg of lysozyme was then added to the suspension, and also 7.5 mg of DNAase to reduce lysate viscosity and thereby to obtain dense precipitates with centrifugation. It was first ascertained that treatment with DNAase and repeated washing by centrifugation did not reduce the rate of succinic acid oxidation. Lysis was allowed to proceed for 30 minutes at 37°. The opalescent yellow lysate obtained was made up to 100 ml with buffer and centrifuged at 22,000 G in the cold. Five washings with cold buffer were carried out by centrifugation. The dark yellow precipitate of cytoplasmic membranes thus obtained, which was free of intact cells, was suspended in 1 ml of buffer; this suspension was used for all enzyme reactions. The suspension was subsequently clarified by the addition of 2 ml of glycerin in order to decrease light scattering. Spectral measurements were made with an SF-4 spectrophotometer.

To further reduce light scattering and to intensify the cytochrome absorption bands, measurement was made with opal glass [5, 6]. Spectra were taken over the range 510-640 mμ at intervals of 2 mμ in the region of the maxima and at intervals of 3-5 mμ over the remaining regions. No measurements were made below this limit because of the large quantity of pigments which impede resolution of the γ-band of cytochromes. Difference spectra were obtained by measurement against an identical sample oxidized by $K_3Fe(CN)_6$.

The concentration of the cytochrome c stock solution was $1.28 \cdot 10^{-7}$ moles/ml [7], with 0.2 ml of an oxidized cytochrome c solution being added to 1 ml of suspension. Concentration of the sodium succinate solution was 2.5 mg/ml of membrane suspension. Nitrogen was determined by the Kjeldahl method and total carbohydrate with anthrone. In the latter case, mannose was used as a standard [8] and color intensity was measured with an FÉK-M colorimeter fitted with a red filter. The membrane preparation comprised 10% of the weight of the original bacteria, and contained 10.25% nitrogen, or 64% protein, and 12.6% carbohydrate.

RESULTS

In order to determine which enzymes of the succinoxidase system are preserved on the cytoplasmic membranes besides succinic dehydrogenase, measure-

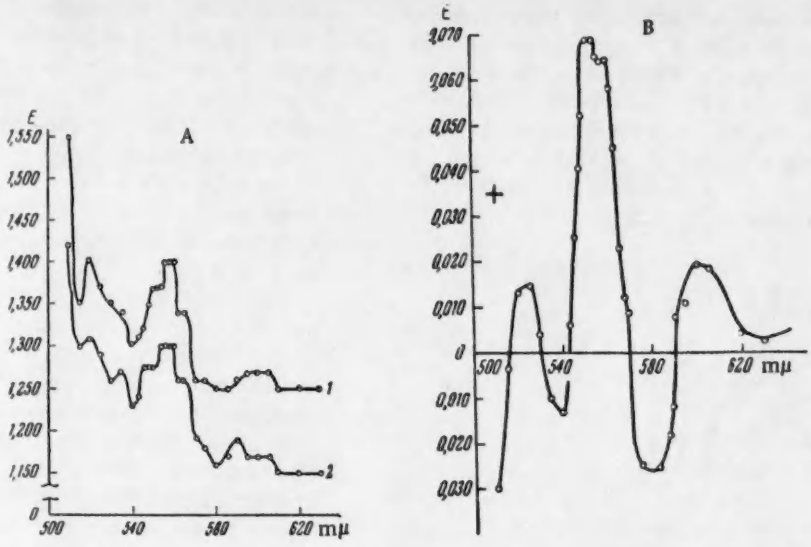


Fig. 1. Absorption spectra of cytochromes of cytoplasmic membranes; cytochromes in the reduced form. A) Control cuvette contained buffer solution; 1) Without succinate. 2) with succinate. B) Control cuvette contained cytochromes oxidized with $K_3Fe(CN)_6$ (difference spectrum).

ments were made of the spectra of cytochromes, which are obligatory components of succinoxidase. In aerobes, cytochromes in intact cells or in preparations of structural elements are usually in an oxidized state; to obtain precise spectra they are reduced by chemical or enzymatic means.

Data from a typical experiment (Fig. 1, A) show that cytochromes b, c, and a, with α-bands at 560, 550, and 590-600 mμ, are present in the membranes; the β-band for b and c is visible at 520 mμ. The broadening of the peaks on the graph is probably due to impurities and to high light scattering by the glycerin preparations, as was also noted by Jackson and Lawton [9].

In this typical experiment, the amount of cytochrome b is somewhat higher than that of c, while cytochrome a is present in the smallest amount. In some experiments, however, the amount of cytochrome c is increased relative to that of cytochrome b (Fig. 1,B), and sometimes they are equivalent in amount.

The impossibility of obtaining preparations of cellular structures which are standard with respect to amounts of cytochromes has been noted by a number of workers [10, 11]. The curves of Fig. 1 show that in the cytoplasmic membranes the cytochromes are partially reduced and that further reduction by succinic dehydrogenase at the expense of succinic acid does not result in an increase in height of the peaks. A similar phenomenon was noted for reduction by hydrosulfite. In experiments in which bacteria were aerated 1-2 hours prior to lysis, membrane preparations contained oxidized cytochromes as a result of exhaustion of endogenous substrates (Fig. 2), and it was then possible to reduce them enzymatically in the presence of succinic acid. A comparison of the curves of Fig. 1 with curve 2 in Fig. 2 shows that the spectrum obtained after enzymatic reduction (Fig. 1) is similar to that in which reduction is complete (Fig. 2, curve 2).

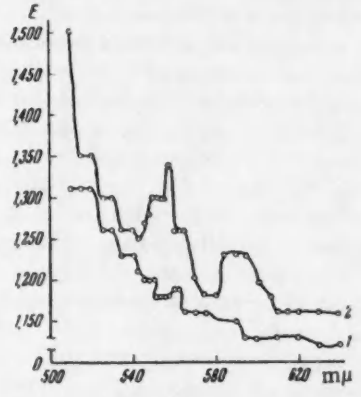


Fig. 2. Absorption spectra of cytochromes of the cytoplasmic membranes; cytochromes in the oxidized form. Control cuvette contained buffer solution; 1) Without succinate; 2) with succinate.

In preparations in which cytochromes are partially reduced, and also in those in which reduction is complete, no subsequent reoxidation of the cytochromes was observed; this may be a confirmation of the assumption previously advanced that the terminal link in the succinoxidase chain is inactivated during lysis. Reduced cytochrome b can be oxidized by addition of oxidized cytochrome c (Fig. 3, 1).

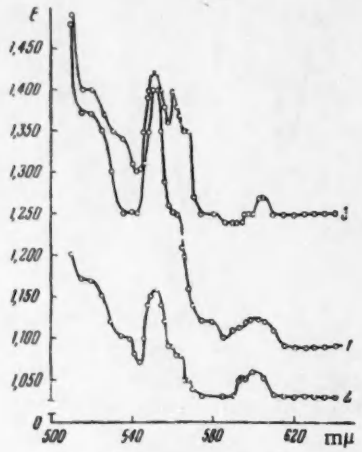


Fig. 3. Oxidation of cytochrome b in the cytoplasmic membranes by cytochrome c. Control cuvette contained buffer solution; 1) In the presence of 0.2 ml of cytochrome c; 2) the same after washing; 3) washed material reduced with hydrosulfite.

The experiment was performed as follows: after membranes were washed four times, 0.6 ml of a solution of oxidized cytochrome c was added to 3 ml of a suspension prepared as described above. The reaction was allowed to proceed 30 minutes at 30°. A 1.2 ml aliquot of the suspension was removed and combined with 2 ml glycerin, a spectrum was then taken (Fig. 3, 1). The remaining 2.4 ml was washed twice by centrifuging in buffer and the residue made up to a volume of 2.4 ml. A few crystals of hydrosulfite were added to 1.2 ml (Fig. 3, 3), and the other 1.2-ml sample served as a control (Fig. 3, 2).

A comparison of the curves of Fig. 3 reveals that cytochrome c actually oxidizes cytochrome b, the α-band of which is not visible either before or after washing out cytochrome c. Cytochrome b remains in the preparation after washing and can be detected upon reduction with hydrosulfite. No changes in the region of absorption by cytochrome a were observed after treatment with oxidized cytochrome c.

DISCUSSION

Micrococcus lysodeikticus is characterized by a high ratio of oxygen absorbed to amount of cytochrome [12], which indicates the presence of an active cytochrome oxidase. According to our earlier data, these bacteria contain a succinoxidase system which is impaired to some degree by lysis. As is well known, succinoxidase includes a number of components, among which are succinic dehydrogenase, cytochromes, and cytochrome oxidase.

In this study it has been shown that in the cytoplasmic membranes, together with succinic dehydrogenase, there are present cytochromes b, c, and a. These observations are in agreement with those of Jackson and Lawton [9] for membranes of Micrococcus lysodeikticus, and also with those of Weibull and Bergstrom [13], who worked with membranes from Bacillus megaterium.

The presence of both succinic dehydrogenase and cytochrome b indicates a stable connection between these two components. Singer and Lara [14, 15] found cytochrome b in purified preparations of succinic dehydrogenase from Propionobacterium pentosaceum. The existence of a simple complex of a flavoprotein and cytochrome b has been noted by Green [16] for the electron transport particles of heart muscle mitochondria and also by Lundegardh [17] for wheat rootlets.

The discovery of reduced cytochromes b and c in the membranes, the identity of their spectra with those obtained after enzymatic reduction of oxidized cytochromes, and the absence of a reoxidation of the reduced cytochromes provide support for the previously-advanced hypothesis that the impairment of action of succinoxidase with cell lysis is due primarily to a disturbance of the cytochrome oxidase component. This is probably also the reason that membranes contain cytochromes in the reduced state. Apparently the oxidation system is disrupted during lysis and the cytochromes are reduced by substrates in the lysate.

The impression arises that this system has lost the capacity to transfer electrons to oxygen. In order to partially restore the effect of the enzyme complex, oxidized cytochrome c was added as an intermediate electron carrier. Because of its high solubility, cytochrome c easily interacts with that part of the succinoxidase complex remaining in the membranes. Under these conditions there is a reduction of exogenous cytochrome c and an oxidation of cytochrome b present in the membranes. It is well known that cytochrome c also oxidizes other enzymes of lower redox potential, in particular, succinic dehydrogenase. This is indicated by preliminary experiments on reduction of cytochrome c by a dialyzed and lyophilized supernatant solution obtained after removal of membranes.

SUMMARY

In the cytoplasmic membranes of Micrococcus lysodeikticus cells cytochromes b, c, and a have been detected. The presence of both cytochromes and succinic dehydrogenase indicates a localization of the entire succinoxidase system in the membranes. Inhibition of succinoxidase activity upon lysis of the cells is presumably

linked with a derangement of the organization of the enzyme complex and an inhibition of the terminal link – – cytochrome oxidase.

LITERATURE CITED

[1] A.I. Oparin, N.S. Gel'man, I.G. Zhukova, and M.A.Lukoyanova, Biokhimiya 23, 909 (1958).*

[2] N.S. Gel'man, I.G. Zhukova, M.A. Lukoyanova, and A.I. Oparin, Biokhimiya 24, 488 (1959).*

[3] N.S. Gel'man, I.G. Zhukova, and A.I. Oparin, Doklady Akad. Nauk SSSR 126, 198 (1959).†

[4] A.I. Oparin, N.S. Gel'man, and I.G. Zhukova, Doklady Akad. Nauk SSSR 105, 1036 (1955).

[5] K. Shibata, J. Biochem. (Jap.) 45, 599 (1958).

[6] K. Shibata, Methods of Biochemical Analysis (Inter. Publ. Inc., New York, 1959), p. 177.

[7] W.W. Umbreit, R.H. Burris, and J.F. Stauffer, Manometric Techniques and the Study of Tissue Metabolism [Russian translation] (IL, Moscow, 1951) p. 335.

[8] A. Gilby, A. Few and K. Mc Quillen, Biochim. et biophys. acta 29, 21 (1958).

[9] F. Jackson and V. Lawton, Nature 181, 1539 (1958); 182, 799 (1958); Biochim. et biophys. acta 35, 76 (1959).

[10] W. James and H. Lundegårdh, Proc. Roy. Soc. B150, 7 (1959).

[11] B. Chance, J. Biol. Chem. 197, 567 (1952).

[12] L. Smith, Bacteriol. Revs. 18, 106 (1954).

[13] C. Weibull and L. Bergström, Biochim. et biophys. acta 30, 340 (1958); C. Weibull, H. Beckman and L. Bergstrom, J. Gen. Microbiol. 20, 519 (1959).

[14] T. Singer and F. Lara, Proc. Symp. Enz. Chem. Tokyo, Maruzen, p. 330 (1958).

[15] F. Lara, Biochim. et biophys. acta 33, 565 (1959). 33, 565 (1959).

[16] D. Green, Harvey lectures. (Acad. Press Inc., New York, 1958); D. Ziegler and K. Doeg, Arch. Biochem. and Biophys. 85, 282 (1959).

[17] H. Lundegårdh Biochim. et biophys. acta 35, 340 (1959).

*Original Russian pagination. See C.B. Translation.

†See English Translation.

A STUDY OF PHOSPHORUS METABOLISM OF THE GREEN PHOTOSYNTHESIZING SULFUR BACTERIUM IN RELATION TO CARBON DIOXIDE FIXATION

V.H. Shaposhnikov and V.D. Fedorov

Department of Biology, M.V. Lomonosov State University, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 487-495,
May-June, 1960
Original article submitted August 26, 1959

Beginning with 1942, the Wisconsin group of microbiologists published a series of studies [1, 2] concerning Thiobacillus thiooxidans. The principally important moment in the results that were obtained was the following conclusion: in the absence of CO_2 the cells of Th. thiooxidans, during oxidation of sulfur, store up energy in the form of energy-rich phosphate bonds by utilizing the orthophosphate of the medium. Such cells, under the conditions that excluded oxidation of sulfur, acquired the ability to fix registerable amounts of carbon dioxide. Such fixation of CO_2 was accompanied by transformation of the esterified phosphate back into orthophosphate, which was then released by the cells into the medium.

However, the studies of Baalsrud and Baalsrud [3, 4] with Th. thiooxidans, Th. thioparus and Th. denitrificans have cast doubt on the previous conclusions. These authors presented results according to which fixation of CO_2 could be observed only during the concurrent presence of thiosulfate and oxygen, i.e., under conditions of normal processes of thiosulfate oxidation by the bacteria. Newburgh [5], by utilizing the isotopes P^{32} and C^{14}, rechecked the results of Vogler and Umbreit He confirmed the ability of Th. thiooxidans cells to fix measurable amounts of carbon dioxide under conditions excluding oxidation of sulfur. However, Newburgh has also observed that the cells that were not previously held under aerobic condition in the presence of sulfur showed the ability toward CO_2 fixation. In this case, fixation of CO_2 by the cells was only somewhat lower. Of special importance in Newburgh's experiments was the observation that during CO_2 fixation there could not be observed any release of inorganic phosphate into the medium. Aubert [6] and Milhaud [7] have likewise observed slight CO_2 fixation by suspensions of Th. denitrificans in the absence of thiosulfate and nitrate.

The concept of Vogler and Umbreit evidently received support in the works of Belyaeva with hydrogen bacteria [8] and in the works of Sorokin with sulfate-reducing bacteria [9].

Less definite results were obtained by a number of authors in experiments with photoautotropes. As early as 1944 Emerson et al. [10] had attempted in experiments with Chlorella pyrenoidosa to observe uptake and esterification of phosphorus in light in the absence of CO_2. However, the observed changes in phosphate content of the medium were so insignificant that on their basis it was impossible to come to any definite conclusions. Likewise unsuccessful were the attempts of Aronoff and Calvin [11] who have observed that light has no noticeable effect on phosphorus metabolism of Chlorella vulgaris. However, the experiments of Gest and Kamen [12] with Phodospirillum Rubrum, Chlorella purenoidosa, Scenedesmus-D_3 have completely and clearly shown that uptake and turnover of phosphorus in photosynthesizing bacteria and water plants was greater in light than in darkness. Somewhat later Wintermans [13] was successful in showing that cells of Chlorella accumulated polyphosphate in the light and in the absence of CO_2. In the absence of CO_2, according to Wintermans, ATP labile P was transferred to polyphosphates. Unfortunately, Wintermans did not attempt to check a second, more controversial part of the proposition of Vogler and Umbreit – the possibility of subsequent fixation of CO_2 in darkness and accompanying release of orthophosphate into the medium. It must be admitted that Umbreit's [14] presentation, concerning the presence of directed tie between CO_2 assimilation and utilization of labile phosphate bonds in photosynthesis, up to the present time did not receive any firm experimental proofs (at least not for photosynthesis, characteristic in type to green plants – with production of oxygen).

Wassink, Tjia and Wintermans [15] obtained more definite results while studying the photosynthesizing purple sulfur bacterium, Chromatium. These authors have observed changes in inorganic P comparable to such changes with Th. Thiooxidans [1].

It was proposed that photosynthesizing bacteria could serve as a suitable subject for solving of the question concerning the possibility of working out in time the processes of photophosphorylation and subsequent carbon dioxide fixation in darkness.

MATERIALS AND METHODS

The work was conducted with a pure culture of the green sulfur bacterium Chlorobium thiosulfatophilum. The culture was isolated from the top mud of the River

Moscow; its physiological characteristics were described in previous work [16]. In the conduct of the experiments, whenever it was necessary to obtain a maximal harvest of cells for biochemical analysis, the cells were cultured on a medium of the following constitution: NH_4Cl, K_2PO_4, 0.01%; $MgCl_2$, 0.05%; $CaCl_2$, 0.02%; NaCl, 0.1%; $NaHCO_3$, 0.5%; $Na_2S \cdot 9H_2O$, 0.15%; $Na_2S_2O_3$, 0.4%. The medium was prepared with distilled water to which were added microelements in the following amounts: Fe^{+++} 50 μg% (in the form $FeCl_3 \cdot 6H_2O$); B, 10 μg% (H_3BO_3); Co, 5 μg% [$Co(NO_3)_2 \cdot 6H_2O$]; Mn, 0.5 μg% ($MnSO_4 \cdot 7H_2O$); Cu, 0.5 μg% ($CuSO_4 \cdot 5H_2O$); Zn, 10 μg% ($ZnSO_4$). The pH of the medium was 7.2. The cultures were grown under anaerobic conditions in a thermostat at 30° with constant illumination.

The comparatively small additions of phosphate to the medium (0.01% instead of the usual 0.1%) were determined by the following calculations. In solving of the stated problem an important role was played by the choice of methodology and, first of all, by successful choice of the method for phosphate determination. It was found necessary to discard a number of methods (Fiske-Subbarow [17], Lowry and Lopez [18], Bosse [19]) which colorimetrically determine phosphate directly in an aliquot of the medium; this was due to the presence in the medium of two factors interfering with the determinations: first, the presence of a large mass of pigmented bacterial cells; and second, presence in the medium of oxidized sulfur compounds, mainly hydrogen sulfate, which made it difficult to make determinations even for parallel samples. The very sensitive extraction method of Weil-Malherbe and Green [20] was utilized. An insignificant modification of the method allowed determination of phosphorus in the samples within the limits of 0.5 - 30 μg/ml. The low content of the introduced phosphate (0.01% KH_2PO_4 - corresponding to 23 μg P/ml) in the medium did not necessitate the dilution of the latter. In every case there were five paralled determinations of phosphorus and from the obtained values an average was obtained. Thiosulfate and hydrogen sulfate were determined by the iodometric method. Carbon dioxide was determined in Haldane's apparatus; the hydrogen sulfate of the medium was tied up to 10% H_2SO_4 with 4% $CUSO_4$.

In the present study suspensions of resting cells were not utilized. All experiments were conducted on a culture of Chlorobium thiosulfatophilum, which was developing in a medium described.

DESCRIPTION OF EXPERIMENTS

Larsen [21] observed slight, but rapid, uptake of CO_2 in darkness, following prior illumination of resting Chlorobium thiosulfatophilum cells in the absence of an electron-donor. For this reason, it was found natural to begin the work with the study of this occurrence. The ability of green sulfur bacteria toward CO_2 fixation in darkness was studied on cultures in: middle of lg - growth phase (I), at the end of lg - beginning of stationary phase (II), and finally in the middle of stationary growth phase (III). The structure of the experiments was as follows: in one part of the culture (A), of known growth phase, the amount of CO_2 in illuminated medium was determined, subsequently light was excluded and the CO_2 content was determined at definite time intervals. From the other part of the culture (B) CO_2 was removed by bubbling nitrogen for 10 min at pH 4.5 - 5. The pH was then brought to 7.2 by a solution of NaOH and light was introduced for 4-18 hrs. Subsequently the culture was placed in darkness, CO_2 was introduced in form of bicarbonate and the CO_2 content was determined at the intervals of 15, 30, 45, 60 min, etc. The obtained results are presented in Table 1.

Fixation of the material. In order to preclude possible changes in the bacterial mass, the culture was fixed rapidly: This was accomplished by adding to one volume of medium four volumes of 96% ethanol. Then the bacterial mass was separated by centrifugation and fixed in 96% ethanol for three hours. Following removal of ethanol the cells were worked over three times with sulfurous ether and dried in a vacuum desiccator over anhydrous gypsum. The material obtained in this fashion was utilized for analysis. In a typical experiment from 170 ml of medium there were obtained approximately 90 mg of fat-free cells.

During the analysis of the bacterial mass the content of phosphorus compounds was determined in the acid-soluble and acid-insoluble fractions.

Study of the acid-soluble fraction. In order to obtain the acid-soluble fraction, the bacterial mass was extracted for 60 min with 2% solution of perchloric acid at 0°. After centrifugation the cells were once again extracted with hydrochloric acid for 30 min. The extracts were pooled together and immediately neutralized with a solution of NaOH, utilizing phenolphthalein as an indicator, and brought to the volume of 15 ml. In the acid-soluble fraction, obtained in the described way, determinations were made of the amount of polyphosphates [22] and of the amount of free nucleotides.

Free nucleotides were determined in the following way. To 10 ml of the acid-soluble fraction there was added 0.1 volume of Carboraffin brand charcoal and were shaken in a shaker for 15 min. The charcoal was removed by centrifugation and washed three times with distilled water. The charcoal-absorbed nucleotides were eluted by shaking the charcoal for 15 min with a solution of 60% ethanol containing 0.5 ammonia. After centrifugation the charcoal was once again washed with the alcohol-ammonia solution and the elutions with nucleotides in them, were pooled together. The obtained solution of nucleotides contained a considerable amount of foreign admixtures which adsorbed UV-light. In order to purify the nucleotides the anionic exchanger Dowex-2 chloride (7% DVB), 300-400 mesh [23] was utilized. To the elution there was added resin in the ratio of 2 mg

to 1 g of the nucleotide, and it was then carefully shaken at room temperature for 30 min. After decantation and washing of the resin once, the nucleotides were eluted with 5 ml of 1 N HCl. The elution was conducted during 20 min with careful shaking. After separation of the first elution, the resin was washed with 3 ml of 1 N HCl for 5 min and the second elution was added to the first. The solution obtained in this fashion contained nucleotides, the amount of which was determined spectrophotometrically (calculation formula [12] given below). The amount of labile-P nucleotides was judged by the amount of orthophosphate, which appeared in the medium following its heating at 100° for 7 min.

Special experiments were conducted in order to clarify the nature of nucleotides of the acid-soluble fraction. The solution of nucleotides obtained after eluting with 1 N HCl (8 ml) was neutralized and concentrated to the volume of 0.5 ml. The concentrated solution was investigated by means of paper electrophoresis [24]. Only one band appeared after development of the electrophoregram in UV-light and its position corresponded to nucleotides of the adenylic system. Bromination of the nucleotide solution [25] and likewise of the elution from the electrophoregram band has shown that the basic, if not the only, system in the acid-soluble nucleotide fraction in Chl. thiosulfatophilum was adenylic system. This allowed, during spectrophotometry, accepting the molar coefficient of extinction as equal to 15000 and the number of nucleotides in millimoles/mole (C) was calculated according to the formula:

$$C = \frac{E_{260} - E_{290}}{15},$$

where E_{260}, E_{290} - extinction at 260 and 290 mμ respectively.

Study of the acid-insoluble fraction. In the remnant, left over after removal of acid-soluble compounds, determinations were made of polyphosphate and nucleotide contents. Hydrochloric acid in the remnant was removed by 96% ethanol, and then the material was extracted for 40 min at 37° with an alcohol-ether mixture (3:1) in order to remove lipids. Alkaline hydrolysis was conducted according to the technique of Schmidt and Thannhauser [26], which was proposed for separation of DNA and RNA. The amounts of DNA and RNA obtained by the alkaline hydrolysis were determined spectrophotometrically [27].

The polyphosphate content of the acid-insoluble fraction was determined by the indirect method: At the end of alkaline hydrolysis and DNA precipitation by acidification of the solution, the contained RNA, polyphosphates etc., were hydrolyzed in 1 N HCl for 7 min at 100°. It was assumed that the whole (if not at least 90%) amount of the orthophosphate, which appeared after hydrolysis, belonged to polyphosphates. Of course, first during alkaline and then during acid hydrolysis some of the nucleic acid phosphorus will become mineralized, and this could give somewhat higher results during determinations of polyphosphates in acid-insoluble fraction nucleotides. This error could be ignored partly because it did not interfere with the solution of the problem and partly due to closeness of the polyphosphate data, as obtained by this method, to previously described data for polyphosphates of the acid-insoluble fraction [22].

RESULTS

In Table 1 are presented results concerning CO_2 content in the culture medium following the transfer of Chlorobium thiosulfalophilum culture from light into darkness.

TABLE 1. Changes in Carbon Dioxide Content in the Medium Following Transfer of the Chlorobium Thiosolfatophilum Culture From Light into Darkness (m moles/ml). A) Light and darkness incubation under conditions of common culture medium. B) incubation in light after removal of CO_2 from the medium and incubation in darkness after addition of bicarbonate. Figures in parenthesis – length of incubation in hours.

Growth phases of the culture	Condition of expt.	In light		In darkness (after addition of $NaHCO_3$ in experiments B) after (minutes)						Maximal uptake of CO_2 in darkness
		after removal of CO_2	before transfer of CO_2 in darkness	0	15	30	45	60	90	
Middle of logarithmic phase	A	–	14.4	–	–	15.0	–	–	15.8	–
	B (18)	0.8	0.4	15.3	15.1	14.9	–	14.8	15.3	0.5
End of logarithmic phase - beginning of stationary phase	A	–	16.2	–	–	16.4	16.5	–	–	–
	B (6)	0.2	0.0	25.6	25.5	25.3	25.1	–	25.5	0.5
	A	–	14.0	–	14.2	14.8	–	–	–	–
	B (15)	0.7	0.1	23.9	23.7	23.5	23.9	24.1	24.3	0.4
Middle of stationary phase	A	–	10.8	–	–	10.9	–	–	–	–
	B (4)	0.1	0.0	31.5	–	31.1	–	31.4	–	0.4
	A	–	7.2	–	7.2	7.4	–	–	–	–
	B (17)	0.3	0.1	18.1	18.0	17.8	17.8	18.1	18.3	0.3

Examination of the data in Table I shows that following the transfer of the culture from light into darkness (experiments A), the CO_2 content of the medium was increased due to its production by the cells. In experiments B (prior illumination of the cultures in the absence of CO_2) transfer of cultures from light into darkness (with introduction of bicarbonate into the medium) caused in the beginning a short-term (15-60 min) drop in the CO_2 content; subsequently the amount of carbon dioxide increased. The indicated lowering of the CO_2 content of the medium could be explained by CO_2 fixation in darkness by the cells of Chlorobium thiosulfatophilum. Comparison of the results obtained in experiments A and B allowed us to conclude that uptake of CO_2 in darkness is observed only in cultures preliminarily illuminated in the absence of CO_2. Besides this, increase in light exposure interval in the absence of CO_2 evidently did not lead to a subsequent increase in CO_2 fixation in darkness. Most marked CO_2 fixation was observed, evidently, in cultures being in the lg-growth phase. However this cannot be completely relied on, since the absolute CO_2 fixation values in all cases were exceptionally small (of the order of 0,5 millimoles/ml).

Experiments concerning phosphorus metabolism in Chl. thiosulfatophilum were conducted on cultures which were at the end of the logarithmic growth phase. The interval of culture illumination, in the absence of CO_2 to the time of their transfer into darkness, as a rule did not exceed seven hours. The only exceptions were those experiments in which special observations were made on the changes in medium orthophosphate during long-term illumination of the bacterial cultures in the absence of CO_2. In this case, the first determinations of medium orthophosphate were made immediately after CO_2 expulsion from the medium and placing of the cultures in light. Subsequent phosphorus determinations, which were made during definite time intervals while the cultures were in light and after they were transferred into the darkness with CO_2, revealed a number of interesting principles (Table 2).

Thus, in light in the absence of CO_2 there was observed a slight but distinct lowering of phosphate in the culture medium (experiments 1 and 3). Under these conditions the major part of phosphate was utilized by the cells during the first 1-2 hours. In subsequent 2-3 hours utilization of phosphate dropped rapidly and then phosphate was released by the cells into the medium (experiments 2 and 3).

On the other hand it is important to point out that at the time of CO_2 fixation in darkness or immediately after cessation of fixation (period of one hour with culture in darkness) there could be observed a release of small amounts of orthophosphate by the cells into the medium. However, in all of the experiments, the amount of orthophosphate released during CO_2 fixation in darkness was always smaller than the amount taken up from the medium during the preliminary illumination of the culture in the absence of CO_2.

The last fact calling attention to itself was the observation that concurrently with utilization of phosphate from the medium by the cells in light and in the absence of CO_2, there took place a slight (in order of mmole/liter oxidation of thiosulfate. The obtained results allowed us to pose the following question: Into what compounds was orthophosphate included when it was absorbed by the cells in light?

In all experiments it was noticed that during the time of illumination, in the absence of CO_2, the cultures began gradually (and in some experiments very rapidly) to acquire a chlorine-color shade, and following prolonged illumination it yellowed. Evidently, the observed yellowing of the culture was in some way related to changes in cellular pigments. Solution of these questions necessitated "a look inside of the cell" and the control of a number of systems and compounds, which could be responsible for the observed changes in the cultures of Chl. thiosulfatophilum;

Earlier during the study of polyphosphates of the photosynthesizing bacterium [22] it was shown that almost all of the phosphorus of the bacteria studied (in Chlorobium thiosulfatophilum - about 90% of the

TABLE 2 Changes in the Intracellular Content of Various Forms of P, Free Nucleoeotides and Nucelic Acid of Chl. Thiosulfatophilum Culture Medium During Transfer from Light Incubation, In the Absence of CO_2, to Darkness With Addition of Bicarbonate to the Medium.

No. of experiment	Substances studied	Time of illumination, in hours						After 1 hr. darkness (in period of CO_2 fixation).
		imm. after removal of CO_2	1	2	3	7	18	
1	P	1,4	1,0	0,9	0,85	—	—	0,95
	$S_2O_3^=$	12,7	—	—	11,9	—	—	—
2	P	5,3	—	—	—	—	5,5	—
	$S_2O_3^=$	12,1	—	—	—	—	10,3	—
3	P	12,1	11,8	11,6	11,4	11,6	—	11,7
	$S_2O_3^=$	9,6	—	—	—	8,0	—	—

intracellular phosphorus, not counting orthophosphate) belonged to polyphosphates and nucleic acids. For this reason in the present work, during the analysis of the bacterial mass, the contents of polyphosphates, nucleic acids and free nucleotides were determined in every case.

Analysis of phosphorus compounds of Chl. thiosulfatophilum at the end of lg-growth phase (a separate experiment) gave the following results (in % of dry weight of fat-free bacterial mass):

Total P	P-acid-soluble			P-acid-insoluble	
	P-ortho-phosphate	P-polyphos-phate	P-ATP	P-poly-phosphates	P-nucleic
1.00	0.20	0.312	0.013	0.105	0.43

Comparison of these data with results in Table 2 shows that a change in phosphate content of 0.5 μg P/ml in 170 ml of the medium should produce an intracellular change in phosphorus content of about 80 -an amount well registered by the chosen method. Eighty micrograms of P in a harvest of 80 mg of bacteria (dry weight) from 170 ml of medium comprises 0.1% P by weight - a quantity which determines well the tendency in the intracellular distribution of phosphorus.

During solving of the basic problem – determination of the sites of phosphorus inclusion during illumination and identification of phosphorus sources during CO_2 fixation in darkness – it was necessary to follow the intracellular distribution of phosphorus in bacteria fixed at critical points of the experiment (Table 3).

It appeared interesting in one case to limit the time of illumination in the absence of CO_2 to four hours (a time interval during which there takes place noticeable utilization of medium phosphate: experiment 1, Table 3), in another case - increase the time of illumination to eight hours (when a certain amount of inorganic phosphate is being released by the cells into the medium: experiment 2, Table 3). Analyses were conducted on the material fixed at the "critical" points (A,B,V, Table 3). In every case in the acid-soluble fraction there were determined polyphosphates, labile ATP phosphorus and the amount of free nucleotides, in the acid-insoluble fraction - the content of polyphosphates and nucleic acids (RNA and DNA).

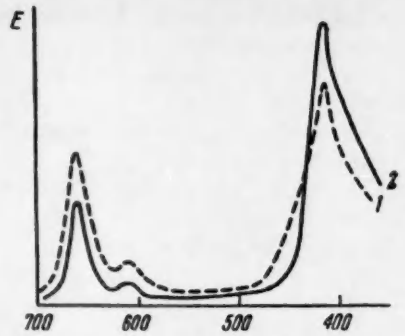
Examination of data in Table 3 shows that during illumination, orthophosphate from the medium was incorporated into polyphosphates of the acid-soluble fraction. During CO_2 fixation in darkness by the cells, there took place some lowering in the polyphosphate content. The level of adenylic system labile P remained unchanged during the observed changes in polyphosphate content.

Comparison of results from experiments 1 and 2 (Table 3) shows that with the increase in culture illumination time in the absence of CO_2, there took place a sharp drop in cellular NA content. Comparison of this fact with some increase in the content of inorganic P in the medium, in experiments with prolonged illumination of the culture (Table 2, experiment 3) lets us assume that autolytic processes began in the culture. It should be mentioned that CO_2 uptake in darkness was observed at the beginning of autolysis, and the latter evidently did not have noticeable influence on the character of changes in the polyphosphate content, under the conditions of the experiment.

Spectrophotometric studies of extinction of the pigments extracted with 80% ethanol from bacterial cultures up to (A) and after (B) illumination of the culture for 4 hrs (experiment 1, Table 3) has shown (Illustration) that during illumination in absence of CO_2 there took place a strong drop in the maximum extinction of chlorobium-chlorophyll (in the region of 660 mμ) with

TABLE 3 Changes in the Intracellular Content of Various Forms of P, Free Nucleotides and Nucleic Acid of Chl. thiosulfatophilum Culture During Transfer From Light Incubation in the Absence of CO_2, to Darkness Incubation with Bicarbonate Added to the Medium. (In % of dry weight of fat-free bacterial mass.)

No.of experiment	Moment of material fixation	Acid-soluble fraction			Acid-insoluble fraction.		
		polyphos-phate -P	ATP - P	free nucleotides	polyphos-phate - P	RNA	DNA
1	A	0,212	0,018	0,135	0,125	3,37	0,80
	B	0,404	0,018	0,136	0,102	3,33	0,83
	C	0,370	0,020	0,139	0,103	3,21	0,89
2	A	0,264	0,026	0,051	0,103	3,43	0,73
	B	0,390	0,021	0,055	0,027	2,00	0,51
	C	0,364	0,022	0,055	0,024	2,00	0,55

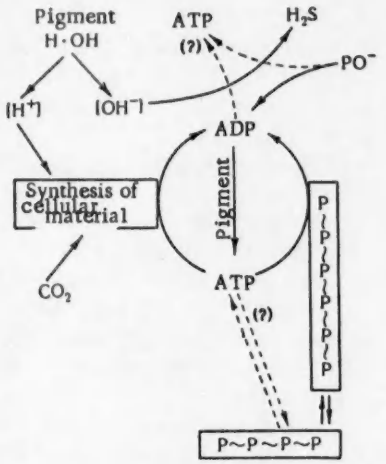


Extinction spectra of Chlorobium thiosulfatophilum pigments extracted with 80% ethanol, prior (1), and after (2), illumination in the absence of CO_2.

a concurrent increase in the maximum of extinction in the region of carotenoids (410-420 mμ).

DISCUSSION

It is convenient to begin the discussion of the results with examination of the proposed scheme for phosphorus participation in the process of bacterial photosynthesis.



The principally important side of the question presented by Vogler and Umbreit was whether phosphorus compounds enter into an energy bond as chemical intermediates between processes of oxidation and carbon dioxide assimilation, and at the present time it is solved in the affirmative formation of energy-rich phosphorus compounds in light (photophosphorylation), as has been shown for the photosynthesizing bacteria by a number of authors [28-30]. Photophosphorylation took place in the presence of oxidized sulfur compounds

This is in good agreement with results of the present experiments, in which uptake of orthophosphate by Chl. thiosulfatophilum cells in the light and in absence of CO_2 was accompanied by oxidation of thiosulfate (Table 2).

Of special interest is the study of participation of polyphosphates in the process of bacterial photosynthesis. The results obtained in the present study, evidently, let us look upon polyphosphates as a reserve of surplus macroenergy bonds (the so called "phosphagens"). It is possible that the conditions of experimental structure expedited the one-sided clarification of the role of polyphosphates in the intracellular phosphorus exchanges. In agreement with presentations developed by a number of laboratories[31, 32], polyphosphates of the acid-insoluble fraction are physiologically active (i.e., take part in synthetic processes), and polyphosphates of the acid-soluble fraction - physiologically inactive. In the present experiments (Table 3) during illumination orthophosphate was incorporated exclusively into polyphosphates of the acid-soluble fraction. This could be explained in the following way: Since in the absence of CO_2 supply, in photoautotropes all cellular synthesis practically ceased, the polyphosphates formed in the acid-insoluble fraction were transferred in the acid-soluble fraction in the form of physiologically inactive polyphosphates. However, lowering of the polyphosphate content in the acid-soluble fraction during CO_2 fixation in darkness, evidently, specifically points to the possibility of rapid mobilization of phosphate-bond energy, by the surviving cell, in the form of physiologically inactive polyphosphates. An impression was created that polyphosphates were some sort of a "difficult-to-utilize" reserve; at least, in darkness, as well as during CO_2 fixation, there was always only a partial destruction of the polyphosphates formed during illumination. It is possible that this "difficulty in utilization" (which was underlined for Chlorella by Wassink [33], who compared the accumulation and utilization of polyphosphates with formation of cellular starch) could be explained by activation by light of the enzyme, that transferred labile P from polyphosphates to ADP.

Absence of changes in the content of ATP labile P agrees with the fact that the adenylic system (ATP-ADP) indirectly not participating in the CO_2 fixation process is, in its own way, a chemical intermediate in the transfer of orthophosphate into polyphosphate:

$$PO_4^- + ADP \rightleftarrows ATP \qquad (1)$$

$$ATP + (PO_3^-)_n \rightleftarrows (PO_3^-)_{n+1} + ADP \qquad (2)$$

At this the equation constant of the reaction (2) must be of a considerable value (higher than one), as a result of which the reaction is displaced to the right. Equation constant of the reaction (1) must be considerably less than one, since the reaction is displaced strongly to the left. Displacement of the reaction (1) to the right is wholly determined by the supply of energy in form of energy taken up by the pigments from light quanta. Thus it is possible, that the change observed above in the state of pigments (see figure) can be looked

upon as a cellular reaction directed toward exclusion of a definite part of pigments from their participation in the reaction of photophosphorylation.

It appears that results obtained in the present study support the principal side of the concept advanced by Vogler and Umbreit in 1942.

SUMMARY

Experiments conducted with a pure culture of the green sulfur bacterium Chl. thiosulfatophilum have shown that CO_2 fixation in darkness (of the order of 0.5 millimoles/ml in 30-60 min) was observed only after a preliminary 4-18 hours illumination in the absence of CO_2. During illumination of the culture in the absence of CO_2, there was utilization of the medium phosphate by the culture, then it was esterified and incorporated into polyphosphates of the acids-soluble fraction. Phosphorylation was accompanied by oxidation of the medium's thiosulfate.

Upon illumination and addition to the culture of CO_2 in the form of bicarbonate (at the moment of CO_2 fixation in darkness) there was a drop in the content of acid-soluble fraction polyphosphates and this was correlated with release, by the culture, of orthophosphate into the medium.

It was shown that in the acid-soluble fraction of Chl. thiosulfatophilum was found only the adenylic system. In the course of phosphorylation in light in the absence of CO_2 and subsequent fixation of CO_2 in darkness, there were no noticeable changes in the content of ATP labile phosphorus. This presumably excluded the possibility of looking upon ATP as a donor of cellular labile P and pointed to the participation of the adenylic system only as a mediator in the transfer of labile P to other systems (primarily to polyphosphates).

Explanation of the observed correlations in intracellular distribution of phsophorus is given in the scheme described above, and it shows the possible participation of phosphorus in the process of bacterial photosynthesis.

LITERATURE CITED

[1] K. Vogler and W. Umbreit, J. Gen. Physiol. 26, 157 (1942).

[2] W. Umbreit, J. Bacteriol. 67, 387 (1954).

[3] K. Baalsrud and K.S. Baalsrud, in: Symposium on Phosphorus Metabolism (Johns Hopkins University Press. Baltimore, 1952), p. 544.

[4] K. Baalsrud, in: Autotrophic Microorganisms, (Cambridge U. Press. London, 1954), p. 54.

[5] R. Newburgh, J. Bacteriol. 68, 93 (1954).

[6] J. Aubert, G. Milhaud and J. Millet, Compt. rend. Acad. Sci. 242, 2059 (1956).

[7] G. Milhaud, J. Aubert and J. Millet, Compt. rend. Acad. Sci. 243, 102 (1956).

[8] M.I. Belyaeva, Scientific Notes, Kazansk U. 114, 3 (1954).

[9] Yu.I. Sorokin, Doklady Akad. Nauk SSSR 95, 661 (1954).

[10] L. Emerson, J. Stauffer and W. Umbreit, Amer. J. Bot. 31, 295 (1944).

[11] S. Aronoff and M. Calvin, Plant Physiol. 23, 351 (1948).

[12] H. Gest and M. Kamen, J. Biol. Chem. 176, 299 (1948).

[13] J. Wintermans, Proc. Koninkl. nederl. akad. wet, 57, 574 (1954).

[14] W. Umbreit, Bacteriol. Revs 11, 157 (1947).

[15] E. Wassink et al., Proc. Koninkl. nederl. akad. wet. 52, 412 (1949).

[16] V.N. Shaposhnikov et al., Mikrobiologia 27, 529 (1958). *

[17] A.N. Belozerskii and N.I. Proskuryakov, Practical Rules for Plant Biochemistry [in Russian] (Sov. Nauka, Moscow, 1951).

[18] O. Lowry and J. Lopez, J. Biol. Chem. 162, 421 (1946).

[19] K. Lohmann and P. Langen, Biochem. Z. 328, 1 (1956).

[20] H. Weil-Malherbe and R. Green, Biochem. J. 49 286 (1951).

[21] H. Larsen, Kgl. norske vid. selskabs. skr. 1, 7, 3 (Trondheim, 1953).

[22] V.D. Fedorov, Doklady Akad. Nauk. SSSR 126, 406 (1959).*

[23] M. de Deken-Grenson and R. DeDeken, Biochim.. et biophys. acta 31, 195 (1959).

[24] B.F. Vanyushin and A.N. Belozerskii, Doklady Akad. Nauk SSSR 127, 455 (1959).

[25] T. Suzuki and E. Ito, J. Biochem. 45, 403 (1958).

[26] G. Schmidt and S. Thannhauser, J. Biol. Chem. 161, 183 (1945).

[27] A.S. Spirin, Biokhimiya 23, 656 (1958).†

[28] A. Frenkel, J. Biol. Chem. 222, 823 (1956).

[29] A. Williams, Biochim. et biophys. acta 19, 570 (1956).

[30] J. Newton and M. Kamen, Biochim. et biophys. acta 25, 462 (1957).

[31] A.N. Belozerskii and I.S. Kulaev, Biokhimiya 22, 35 (1957).†

[32] P. Krishnan, S. Damle and V. Bajaj, Arch. Biochem. and Biophys. 67, 35 (1957).

[33] E. Wassink, in: Research in Photosynthesis, p. 333 (Interscience Publ. Inc., New York, 1957)

Original Russian Pagination. See C. B. translation

THE OXIDATIVE FUNCTION OF PEROXIDASE FROM CABBAGE (Brassica oleracea L.)

T.M. Ivanova and B.A. Rubin

A.N. Bakh Institute of Biochemistry, Academy of Sciences USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 496-504, May-June, 1960

In a recently published work [1] it was announced that tissues of the white-head cabbage contained considerably high quantities of tanning substances: in that number were free polyphenols. The latter were basically represented by phloroglucinol, i.e., a compound with meta-placed hydroxyls. From among a number of polyphenols, cabbage tissue was able to oxidize, in the presence of atmospheric oxygen, only one polyphenol, phloroglucinol.

Since cabbage tissues do not possess a polyphenol oxidase – an enzyme oxidizing polyphenols with the help of molecular oxygen – it was of interest to study in greater detail the question concerning oxidation of phloroglucinol.

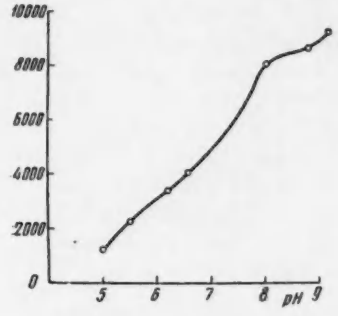


Fig. 1. Influence of pH on phloroglucinol oxidation by cabbage tissue.

METHODS

Living plant tissues, as well as isolated enzyme preparations, were utilized. It was established that the ability of cabbage tissue to oxidize phloroglucinol was at a maximum at alkaline pH (Fig. 1). But since under these conditions polyphenol oxidation could be accomplished by nonenzymatic means, it was decided to conduct the study at a pH inherent to cabbage tissue.

Enzyme activity was determined by measuring oxygen consumption in a Warburg apparatus at 30° in 0.066 M phosphate buffer, at a pH of 6.24. Substrate concentration – 0.01M. Peroxidase activity was determined by the method of Boyarkin [2]. Enzyme activity was calculated in terms of units per 1 g of wet weight of tissue studied, or per 10 mg of enzyme preparation.

Besides a commercially available peroxidase (Lait) the following enzyme preparations were utilized: a preparation of potato [3] polyphenol oxidase (PPO), preparation of horseradish peroxidase [4] and an enzyme preparation from cabbage. A micromethod was utilized for the determination of enzyme protein [4].

In order to isolate the phloroglucinol-oxidizing cabbage enzyme, it was first of all necessary to study the localization of this enzyme in structural components of the cell. Fractionation was conducted by the method of differential centrifugation. A homogenate was prepared from cabbage leaves together with phosphate buffer, pH 7.1, and glucose; it was freed from nuclei, cellular wall particles, etc., by centrifugation at 25 g. Plastids were isolated at 3000 g, mitochondrial fraction - 10,000 g.

Determination of phloroglucinol oxidase (PO) activity in different cellular fractions gave the following results:

Fraction	Microliters of consumed O_2/g per hr	Percent in terms of original solution
Original solution	2017	100
Plastids	24	1.2
Solution after plastid removal	1956	97
Mitochondria	18	0.9
Solution after mitochondrial removal	1805	90

These data show that plastids and mitochondria accounted only for 2.1% of total enzyme activity. It cannot be denied that even this insignificant activity could have been accomplished by incomplete removal of cytoplasm from the plastid and mitochondrial surface. After establishing that the major part of the enzyme was in a soluble state, the enzyme was isolated in the following way.

The cabbage was minced in a meat grinder, the mash was squeezed out and thrown away. The obtained juice was centrifuged at 3000 g for 20 min. The centrifugate was precipitated with two volumes of chilled 80% ethyl alcohol. A light yellow protein precipitate appeared after the solution was refrigerated overnight. The supernatant was decanted, the precipitate was centrifuged in cold, thoroughly washed with chilled solution of ethyl alcohol and then with sulfurous ether, and then dried in a vacuum.

Washing of the precipitate with sulfurous ether, is an especially important operation, for it removes well the free polyphenols and, evidently, products of their oxidation. After washing, the ether acquired a bright yellow coloring, characteristic of phloroglucinol oxidation products [5].

RESULTS AND DISCUSSION

From the data presented in Table 1, it is evident that the enzyme preparation prepared by the above-described method was free of ascorbic acid oxidase and cytochrome oxide, enzymes which are characteristic for cabbage tissues [6]. The isolated enzyme preparation, as well as cabbage suspensions, were unable to oxidize various polyphenols, with the exception of phloroglucinol, a polyphenol which has its hydroxyls located in meta position.

Since the method employed excluded the possibility of participation by ascorbic acid oxidase and cytochrome oxidase in phloroglucinol oxidation, it could be stated that cabbage tissues contained a specific oxidase, oxidizing phloroglucinol. Such a conclusion was supported by the observations of Popov [5], who observed oxidation of phloroglucinol by acetone preparations from tea leaves, preparations of potato and mushroom (Boletus scaber) polyphenol oxidase. Suzuki [7] has likewise described oxidation of phloroglucinol by a polyphenol oxidase preparation from earth roots of Scopalia japonica. Together with this, as a result of a number of investigations, it was accepted as proven that cabbage tissues did not possess a polyphenol oxidase [6, 8].

In agreement with the views of Kursanov and Zaprometova [9], polyphenols with meta-placed hydroxyl groups (phloroglucinol is one of these) are inert in respect to oxidation-reduction changes and partly stable in relation to oxidative enzymes. These authors proposed that oxidation of phloroglucinol in plant tissues occurs via orthoquinones, which could be formed from phenols present in the plants. One of such phenols could be

TABLE 1. Oxidation of Various Substrates by a Cabbage Suspension (A) And an Enzyme Preparation (B)

Substrates	O_2 uptake in μ l per	
	g. hr(A)	10 mg hr (B)
Ascorbic acid	727	0
Cytochrome and hydroquinone	170	0
Hydroquinone	0	0
Resorcinol	0	0
Gallic acid	–	0
Chlorogenic acid	0	0
Pyrogallol	0	0
Pyrocatechol	0	0
Phloroglucinol	1659	599

pyrocatechol, some representatives of which (caffeic and chlorogenic acids) are widely distributed in the plant kingdom.

Insignificant amounts of chlorogenic and caffeic acids were observed in cabbage tissues [1]. Special experiments were conducted in order to clarify the possibility of orthoquinone participation in phloroglucinol oxidation by cabbage tissues. Results of these experiments are shown in Table 2.

Data in Table 2 show that added pyrocatechol and chlorogenic acid had no influence on respiration of the living tissue, but completely inhibited phloroglucinol oxidation. These facts leave no doubt that, in cabbage tissues phloroglucinol oxidation was accomplished without the participation of orthoquinone, and that the latter acts as an inhibitor of meta-phenol oxidation. It should be noted that phloroglucinol oxidation by cabbage enzyme preparation was likewise inhibited by pyrocatechols and chlorogenic acid.

Oxidation of phloroglucinol by cabbage slices and by enzyme preparation isolated from it was depressed by inhibitors of metal-containing enzymes (Table 3). This was expressed very rapidly by sodium azide and by sodium diethyl dithiocarbamate (DDC-Na), which, as is well known, depresses only the activity of copper-containing enzymes. Popov [5] has observed DDC-Na inhibition of phloroglucinol oxidation by acetone preparation from tea leaves.

In this fashion, the data from inhibitor experiments likewise let one suppose that the isolated oxidase was a copper-bearing enzyme, and, more precisely, a polyphenol oxidase (PPO), for it was not endowed with ascorbic acid oxidase activity (Table 1). However, to make such a conclusion was considered premature. First of all, in difference to ascorbic acid oxidase and cytochrome oxidase, which have narrow specificity toward substrates, polyphenol oxidase is less specific and is able to oxidize not only a series of phenols, but also their precursors. (Absence of strict substrate specificity for

TABLE 2. Oxidation of Various Substrates By Cabbage Slices (A) and An Enzyme Preparation (B)

Substrates	O_2 uptake in µl per	
	g · hr (A)	10 · hr (B)
Without substrate (control)	86	0
Pyrocatechol	82	0
Chlorogenic acid	85	0
Phloroglucinol	572	354
Phloroglucinol + pyrocatechol	76	0
Phloroglucinol + chlorogenic acid	89	0

PPO was shown by Shubert [10] for tea-PPO, by Waggoner and Dimond [11] for potato - and tomato - PPO Second, the isolated preparation, besides phloroglucinol oxidation, was able to oxidize polyphenols in the presence of hydrogen peroxide, i.e., it possessed peroxidase activity. Due to the fact that the nature of the isolated enzyme is still unclarified, this enzyme will be referred to as cabbage enzyme preparation (CEP).

A question arose as to whether the isolated enzyme preparation was a mixture of two oxidative enzymes with separate protein and active groups (specific metaphenolase and peroxidase) or whether it was one catalytically active protein, possessing two or more active groups.

It was also necessary to check as to what degree was phloroglucinol oxidase (PO) activity distributed in plant tissues and whether it was correlated with peroxidase activity. For this reason concurrent determinations of PO and peroxidase activities were carried out in a number of plant objects. Results of these experiments are shown in Table 4.

Table 4 shows that the presence or absence of PO activity, as a rule, followed the presence or absence of peroxidase activity in the tissue. Together with this, the relation between the two named enzyme functions varied in different plants.

Relatively high phloroglucinol oxidation activity was possessed by cabbage, horseradish and cucumber tissues, i.e., by such plant tissues which, according to the concept of Szent-Gyorgyi, belong to the peroxidase [12]. High PO activity was also observed in the green parts of the plants.

Attention should be directed to the relatively high level of phloroglucinol oxidation by young barley sprouts, which, according to the data of Kolesnikova and Emenova [13], do not possess a polyphenol oxidase. The highest peroxidase activity was observed in horseradish and the colored part of lemon peel, only in geranium leaves and in onion bulbs were the two enzyme functions absent. Thus, in all of the plant tissues studied, the ability to oxidize phloroglucinol in the presence of atmospheric oxygen was observed together with peroxidase activity.

It is known that enzyme activity of plant objects is not stable, but changes, depending on a whole row of conditions (age, conditions of cultivation and others). Growth changes in PO and peroxidase activity were studied in wheat leaves during seed germination. For these experiments wheat was germinated on filter paper at room temperature. The data are presented in Table 5.

First of all, it should be noticed that during the whole observation period the peroxidase and PO activities were high, and both of these functions were activated with the growth of the leaves. Characteristically, peroxidase and PO activities in the leaves of wheat, during its germination, were increased synchronously to a sufficiently high degree. Thus, in comparison with five-day seedlings, in the 10-day seedlings it was increased by 70-80%, and in the 20-day ones by 141-142%. As the result, during the whole duration of the experiment there was no change in the relation between PO and peroxidase activities.

It is of interest to compare the data from present observations with data from the literature. It is known, for example, that Waygod [14] and Kiraly and Farkas [15] were not able to find polyphenol oxidase in wheat

TABLE 3. Action of Inhibitors on Phloroglucinol Oxidation By Slices and Enzyme Preparation From Cabbage

Inhibitor	Concentration of inhibitor M	Cabbage slices		Cabbage preparation	
		µl O_2 utilized/g per hour	% inhibition	µl O_2 utilized/g per hour	% inhibition
Without inhibitor	–	474	–	814	–
DDC-Na	0.005	119	75	121	85
NaN_3	0.005	56	88	110	86

TABLE 4. Phloroglucinol Oxidase and Peroxidase Activities of Various Plant Objects

Plant Objects	PO activity in μl O_2 uptake/g hour	Peroxidase activity, in relative units (1 g)
Cabbage (head leaves)	2251	35
Horse radish (roots)	1470	250
Cucumber (fruit)	918	30
Potato (tuber)	98	22
Radish (fruit)	47	78
Lemon (peel)	31	166
Orange (peel)	78	60
Onion (bulb)	0	0
Barley (10-day old sprouts)	1149	65
Wheat (10-day old sprout leaves)	376	92
Onion (tops)	860	5
Tea (leaves)	675	31
Sugar beet (leaves)	395	89
Potato (leaves)	379	21
Cotton (leaves)	128	71
Geranium (leaves)	0	0

TABLE 5. Changes in PO and Peroxidase Activity of Wheat Leaves During Sprouting

Days of sprouting	PO activity		Peroxidase activity		
	μl O_2 utilized /g hour	%	relative units (g)	%	PO activity peroxidase activity
5	208	100	54	100	3.9
7	216	104	52	97	4.2
10	376	180	92	170	4.1
13	444	214	104	193	4.2
15	422	203	104	193	4.1
20	504	242	130	241	3.9

leaves. Sokolova and Savel'eva [16] have shown that PPO activity of wheat leaves appeared at low experimental temperatures. It was found recently by Kiraly [17] that leaves from rust-resistant wheat could oxidize gallic acid. Determination of phloroglucinol and gallic acid oxidation (μ liters O_2 uptake /g per hr) by suspensions of wheat leaves in the present experiments gave the following results:

Substrate	Expt. I	Expt. II
Gallic Acid	58	112
Phloroglucinol	348	504

Thus, the ability of wheat leaves to oxidize gallic acid was 5-6 times lower than the ability to oxidize phloroglucinol.

Working with wheat leaves, determinations were made of the dependency of PO and peroxidase activity on pH. It was determined that, with an increase in pH from 4.94 to 6.24, both enzyme functions were increased approximately two-fold. There again attention is called to the fact the intensification of activity occurred in both cases with equal intensity - synchronously. All of these facts serve as the basis for the proposition that peroxidase activity and the ability to oxidize phloroglucinol are characteristics inherent to the same enzyme. Experimental study of this principally important question necessitated foregoing work with the living cell and to transfer the study to isolated enzyme preparations.

The study of the properties of the enzyme obtained from cabbage tissues was initiated by comparing it with potato PPO preparation, horseradish, peroxidase and peroxidase purchased from Lait (crystalline peroxidase).

Figures in Table 6 show that the potato enzyme preparation oxidized a number of polyphenols, among them phloroglucinol, which was oxidized at a rate second only to pyrocatechol.

It was of interest that peroxidase isolated from horseradish and crystalline peroxidase were quite similar to one another. Both of these preparations, as with the cabbage enzyme preparation (Table 2), oxidized, from among the substrates tried, only phloro-

TABLE 6. Oxidation of Various Substrates by Enzyme Preparation, Uptake in μl O_2 per 10 mg per Hour

Substrates	Potato PPO	Horse-radish peroxidase	Crystalline peroxidase
Ascorbic acid	–	0	0
Gallic acid	26	0	0
Chlorogenic acid	40	0	0
Pyrogallol	125	0	–
Pyrocatechol	301	0	0
Resorcinol	–	0	–
Phloroglucinol	213	418	917

Enzyme preparation	PO activity in uptake of O_2/10 mg/hour		Peroxidase activity in relative units (10 mg)	
	for preparation	for protein	for preparation	for protein
CEP (cabbage enzyme preparation)	1043	3032	139	405
PPO - potato	383	–	25	–
Peroxidase - horse radish	548	–	236	–
Crystalline peroxidase	903	1521	740	1246

glucinol; the intensity of the latter's oxidation by preparations from cabbage, horseradish and also by crystalline peroxidase, were quite pronounced. The ability to oxidize phloroglucinol was correlated in all of the preparations studied with peroxidase activity (calculations of enzyme activity in the given experiments were conducted on 10 mg of preparation and on 10 mg of its protein).

Continuing the comparative studies on the properties of enzyme preparations, determinations were made of changes in PO and peroxidase activity in CEP and crystalline peroxidase in relation to changes in medium pH. For this purpose, 0.066M phosphate buffer was utilized. Results of these determinations are presented in Fig. 2.

From the literature it is known that peroxidase activity remains unchanged within the pH limits of 4.0 and 11.0 [18]. The data obtained by the present authors have likewise shown that peroxidase properties of CEP and of crystalline peroxidase remained practically unchanged within the pH limits of 4.0 and 8.0 (Fig. 2). Characteristic pH-dependency curves were obtained for both preparations during phloroglucinol oxidation. Optimum pH in both cases was at about 7.8. During elevation of the pH from 5.0 to 7.0, phloroglucinol oxidation was increased four-fold by CEP, as well as by living cabbage tissues (Fig. 1).

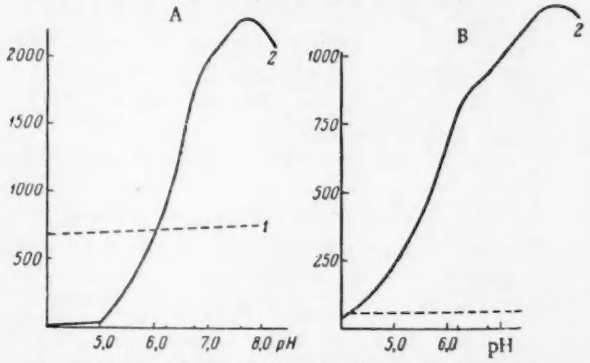


Fig. 2. Influence of pH on peroxidase and phloroglucinol oxidase activity of enzymes. A. Crystalline peroxidase B. Enzyme preparation from cabbage 1) Peroxidase activity in relative units (10 mg); 2) Phloroglucinol oxidas activity in μl O_2 utilized/10 mg per hour.

From the curves in Fig. 2 it is also evident that PO activity of the crystalline peroxidase was not exhibited at a pH lower than 5, and only at a pH value higher than this was the enzyme capable of oxidizing phloroglucinol. In the CEP this ability was exhibited at a lower pH. It is possible that the high PO activity of the enzyme in the alkaline region was related to changes in the valence of hematin, which, according to data in the literature [18], could take place in plant peroxidase in an alkaline medium.

The data brought out above show that the enzyme isolated from cabbage had many properties in common with peroxidases. In difference to potato PPO, the present preparations oxidized from among the polyphenols only one, phloroglucinol. Besides this, the CEP possessed peroxidase activity. Peroxidase activity of both enzymes was not dependent on pH of the medium. Phloroglucinol oxidation by these enzymes was characterized by one and the same dependency on hydrogen ion concentration. The reaction optimum for both enzymes was found in the alkaline region at about 7.8. In an acid medium PO activity was not affected for CEP or for crystalline peroxidase.

Everything that has been reported above permits us to make the proposition that phloroglucinol oxidation by living tissue and enzyme preparations was one of the functions of peroxidase catalytic activity. This question needs further and more thorough study.

Bakh and Vilenskii [19] have found that peroxidases possess relatively high thermostability. This was further supported by other investigators [20]. It was established that peroxidase within plant objects was inactivated more rapidly than peroxidase isolated in form of a preparation [21]. Enzyme preparations isolated in the present study were likewise stable toward heating. Complete inactivation necessitated not less than 10 minutes of boiling. Concurrently an interesting property of crystalline peroxidase was observed. At the time that phloroglucinol oxidation and peroxidase properties of horseradish and cabbage enzymes, as well as potato PPO, were inactivated by 10-minute boiling, the crystalline peroxidase retained about 9-10% of the original activity. Phloroglucinol oxidation intensity of crystalline peroxidase was not reduced after boiling, but

TABLE 7. The Influence of 10-minute Boiling on Phloroglucinol Oxidation and Peroxidase Activity of Enzyme Preparations

Enzyme preparation	Phloroglucinol oxidation in μl O_2 utilized/10mg/hour		Peroxidase activity relative units (10 mg)	
	without boiling	with boiling	without boiling	with boiling
Potato PPO	223	0	25	0
CEP	544	0	139	0
Horseradish peroxidase	548	0	236	0
Crystalline peroxidase	908	1324	684	62
Crystalline peroxidase	969	1360	740	77
Crystalline peroxidase	922	1301	768	71

on the contrary, it was increased by more than 40% (Table 7).

From the curves in Fig. 3 it is evident that the increased ability of crystalline peroxidase to oxidize phloroglucinol was preserved even after boiling for 60 min. With further boiling, intensity of phloroglucinol oxidation decreased. Even after boiling for 120 min, crystalline peroxidase was still able to oxidize phloroglucinol, whereas peroxidase activity disappeared after boiling for 75 min.

It could be proposed that phloroglucinol oxidation under the given conditions was catalyzed by hematin, which was freed following denaturation of the protein carrier. Such a proposition is more in place, because iron-porphyrin compounds were found in plants [22], and because these compounds could play an important

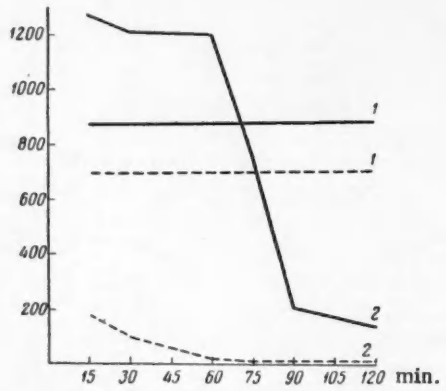


Fig. 3. Influence of Boiling on The Activity of Crystalline Peroxidase
1. Enzyme activity without boiling
2. Enzyme activity after boiling
Broken line – peroxidase activity in relative units. Solid line – phloroglucinol oxidative activity in μl O_2 utilized /10mg hour.

catalytic role in living organisms. This question, likewise, requires further study.

In accordance with presently accepted views, peroxidase functions in plants are directed toward oxidation of various polyphenols and aromatic amines at the expense of activated oxygen from peroxides; in the presence of molecular oxygen alone, peroxidase is inactive. However, a number of facts and observations during the past few years do not agree with this point of view and attest the fact that peroxidase is not completely devoid of properties characteristic of oxidases. For example, spectroscopic observations show that the trivalent iron of peroxidase, which in reaction with peroxide does not change valence, can, under influence of a strong reducing agent (hydrogen sulfate) change to a bivalent [18]. Besides, it has been established that in some plants peroxidase can function as an oxidase of hydroxymalenic acid. During this reaction the valence of peroxidase iron was likewise changed (iron was reduced) [23].

The above-mentioned data from the present observations give a base for the proposition that one of the functions of peroxidase is oxidation of phloroglucinol (and possibly of other phenols) at the expense of inactive molecular oxygen. In other words, peroxidase can function as a phenoloxidase.

Taking into account the complexity and the multicomponency of peroxidase [24, 25], it should be acknowledged that in order to decipher the mechanism of the observed phloroglucinol oxidation by cabbage peroxidase, there is need for further, more intense study, which is being conducted by the authors at the present time.

SUMMARY

An enzyme preparation was isolated from cabbage which was responsible for phloroglucinol oxidation and which did not possess ascorbic acid oxidase and cytochrome oxidase activity.

Cabbage slices oxidized phloroglucinol; this oxidation was inhibited by specific inhibitors, which de-

press activity of metal - enzymes. Tissue PO activity was concentrated in cellular cytoplasm. Phloroglucinol oxidation by living cabbage tissue as well as by enzyme isolated from it could take place directly at the expense of atmospheric oxygen, without orthoquinones participation. Together with this, the enzyme preparation was able to oxidize polyphenols with the help of peroxide oxygen, i.e., it had peroxidase activity.

In a number of objects it was established that the ability of plant tissues toward phloroglucinol oxidation was correlated with peroxidase activity.

The ability toward phloroglucinol oxidation at the expense of molecular oxygen was discovered for a crystalline enzyme preparation (Lait), as well as for the presently isolated preparation from horseradish.

The presented facts let us propose that with respect to phloroglucinol and some other substances, peroxidase can function as an oxidase - an enzyme capable of activating molecular oxygen. The question concerning the mechanism of this reaction is being studied.

The authors wish to express their gratitude to M.A. Davydova for participation in the experimental part of this study.

LITERATURE CITED

[1] B.A. Rubin and T.M. Ivanova, Doklady Akad. Nauk SSSR 125, 213 (1959). *

[2] A.N. Boyarkin, Biokhimiya 16, 352 (1951).

[3] Szent-Gyorgyi, Biochem. Z. 162, 399 (1925).

[4] A.N. Belozerskii and N.I. Proskuryakov, Practical Guide to Plant Biochemistry [in Russian] (Sov. Nauka, Moscow, 1951).

[5] V.R. Popov, Dissertation: Oxidative Processes in Production of Tea [in Russian] (Moscow, 1952).

[6] B.A. Rubin and E.P. Chetvepilova, Izv. Akad. Nauk SSSR (Sev. Biol.) No. 4, 120 (1951).

[7] V. Suzuki, Bot. Mag. 68, 227 (1955).

[8] V.H. Bukin, Dissertation Ascorbic Acid Oxidation in Plant Tissues [in Russian] (Moscow, 1941).

[9] A.L. Kursanov and M.N. Zapeometov, Biokhimiya 14, 467 (1941).

[10] T.A. Shubert, Dissertation Phenolase System and Its Role in Respiration and Fermentation of Tea [in Russian] (Moscow, 1951).

[11] P.E. Waggoner and A.E. Dimond, Phytopathol. 46, 495 (1956).

[12] A. Szent-Gyorgyi, Biokhimiya 2, 152 (1937).

[13] P.A. Kolesnikov and S.V. Emenova, Fiziologiya Rastenii, 3, 480 (1956).

[14] E. R. Waygod, Canad. J. Res. 28, 7 (1950).

[15] Z. Kiraly and G.L. Farkas, Arch. Biochem. and Biophys. 66, 474 (1957).

[16] V.E. Sokolova and O.N. Savel'eva, Doklady Akad. Nauk SSSRIII, 165 (1956).

[17] Z. Kiraly, Phytopathol. Z. 35, 23 (1959).

[18] D.M. Mikhlin, Biological Oxidation [in Russian] (Izd. AN SSSR, Moscow, 1956).

[19] A.N. Bakh and V.A. Vilenskii, Collection: Works in Chemistry and Biochemistry [in Russian] (Izd. AN SSSR Moscow, 1950), p. 622.

[20] H. Theorell and A. Mahley, Acta chem. scand. 4, 422 (1950).

[21] F. Herblinger and F. Kiermeier, Biochem. Z. 318, 411 (1958).

[22] D. Keilin and E.F. Hartree, Proc. Roy. Soc. London 98 B, 312 (1925).

[23] B. Swidin and H. Theorell, Nature 145, 71 (1940).

[24] H. Theorell, Arkiv for Kemi, Mineralogi och Geologi 16 A, 3 (1942).

[25] M.A. Jarmin and R. Thomas, Biochem. J. 56, 631 (1954).

*See English translation.

CHANGES IN SOME PROPERTIES OF MYOSIN, ACTOMYOSIN, AND ACTIN UNDER THE INFLUENCE OF HIGH PRESSURE

I.I. Ivanov, Yu.N. Bert and N.A. Lebedeva

Department of Biochemistry, Leningrad Pediatric Medical Institute

Translated from Biokhimiya Vol. 25, No. 3, pp. 505-510, May-June, 1960
Original article submitted August 28, 1959

We showed previously [1, 2] that myosin dissolved in 0.6 M KCl under the influence of high pressure (on the order of 4000 atm), though it keeps its hydrophilic properties, can dissolve in salt media with high ionic strength and precipitate on dialysis against pure water, while it fully loses ATP-ase activity and the ability to form complexes with actin with formation of the contractile protein, actomyosin. It was also shown that the viscosity of myosin which did not contain marked contaminants of actomyosin increased considerably under high pressure.

Actomyosin gels submitted to the action of high pressure lose the ability to react specifically on ATP, that is, to decrease the viscosity in 0.6 M KCl on the addition of ATP or to undergo syneresis in Szent-Gyorgyi solution.

In the present work we have secured more complete results on the properties of the enzymatically inactive form of myosin obtained under the action of high pressure which in the future for brevity we will call baromyosin. We also considered it necessary to study in more detail the conditions under which inactivation under pressure begins both for myosin and for its complex with actin.

METHODS

The protein was isolated from the tail muscles of rabbits killed by air embolism. After exsanguination the muscles were cooled, freed from connective and fatty tissues, and twice passed through a cooled meat grinder.

Myosin was isolated as described previously [2]. The myosin solution was kept at 1-3°. It was observed that decrease in enzymatic activity of myosin during keeping depended on the concentration of the protein in the solution. At concentrations of 1-1.3% with ten days keeping at 1-3°, the ATP-ase activity did not decrease more than 30%; at concentrations of 0.04% a sharp fall in ATP-ase activity was sometimes found even on the following day.

The purity of the preparations was tested by measuring the viscosity of the solutions before and after addition of ATP in an Ostwald viscosimeter. In our preparations the viscosity of the proteins on addition of ATP either did not fall, or fell by not more than 3-8%.

Actin was obtained according to Straub [3]. A working solution of actin was obtained as needed by water extraction of "acetone muscle powder". For activation, KCl and $MgCl_2$ were added to the water solution of actin to final concentrations of 0.6 and 0.0005 M respectively.

Actomyosin was obtained by the method of Szent-Gyorgyi [4]. The homogenate, after removal from it of water soluble proteins, was treated with five times its volume of Weber solution and extracted for 24 hours in the cold with periodic mixing. The resulting viscous solution of actomyosin was squeezed through a linen bag or four layers of gauze and precipitated with ten times the volume of twice-distilled water; the pH of the solution reached 6.8. The precipitate of actomyosin was separated by centrifuging and dissolved in KCl to a concentration of 0.6 M.

ATP-ase activity was determined by the usual method, by the increase in inorganic phosphorus in the sample and was expressed in μg P/mg N after 5 min, or in Q_p. Composition of the sample: 1 ml protein solution (0.04-0.07 mg N), 1 ml borate buffer, pH 8.6, 0.5 ml 0.03 M $CaCl_2$, and 1 ml of 0.5% ATP solution.

The sample was incubated for 5 min at 37°. Reaction was stopped by addition of 0.5 ml of 20% TCA. The content of inorganic phosphate in the filtrate was determined by the Fiske-Subbarow method. Parallel control experiments were run, in which TCA was added before incubation with ATP. The Q_p of the isolated myosin preparations varied in the limits 900-2500, of actomyosin, 700-800. The relative viscosities of the protein solutions were determined in an Ostwald viscosimeter at 0°. The time of outflow of the experimental solutions was compared with the time of outflow of a 0.6 M aqueous solution of KCl.

Experiments using high pressure. Myosin and actomyosin underwent pressure in a high-pressure press described in the work of Bresler et al. [5]. A volume of 4-6 ml of the studied solution was poured into a plastic bag which was carefully tied up and placed in cylinder filled with

water. The protein in all cases except those in which the effect of prolonged action was studied was submitted to pressure for ten min. Molec. wt. was determined by light scattering and in the ultracentrifuge.

RESULTS

Change in Molecular Weight of Myosin

Three samples of myosin were studied. It was shown that the molecular weight of myosin after the action of 4000 atm pressure increased 1.5-2 times. Thus, for example, the molecular weight of myosin in 1% solution, determined by light scattering, was 760,000. After the action of 4000 atm pressure the molecular weight rose to 1,600,000 (these results are given without correction for the asymmetry of the macromolecule).

In the ultracentrifuge the sedimentation constant ($S_{20,w}$) of an 0.33% myosin solution before the action of pressure was 5.92 Svedberg units. The action of 4000 atm pressure was carried out on an 0.44 and a 0.87% solution of myosin which then for the determination were diluted to 0.33%. The values of S for these samples of baromyosin were 9.57 and 10.66 Svedberg units respectively, which shows an increase in molecular weight of the protein particles. This agrees well with the increase under the action of pressure of the viscosity of solutions of these types of myosin by 1.5 times.

Thus, on partial denaturation of myosin caused by raised pressure as in heat denaturation [8, 9] aggregation of the myosin molecule obviously occurs.

Change in Viscosity of Myosin

The greatest increase in viscosity was found at pressures of 3500-4000 atm. Increase in viscosity under pressure was especially noticeable in cases of high concentration of protein in the solution; thus, the viscosity of a 0.5% myosin solution doubled under a pressure of 3500 atm, and a 1.44% myosin solution quadrupled.

Change in ATP-ase Activity of Myosin

In this series of experiments we studied the action of pressures over the range 500-4000 atm. Myosin solutions of different concentrations did now show exactly the same sensitivity with respect to the action of high pressures. The greatest stability of myosin ATP-ase appeared in solutions with a relatively high protein concentration. Thus, at concentrations of 2 mg N/ml full inactivation of ATP-ase appears at 3000 atm. On this, the ATP-ase activity falls gradually with changing pressure. At concentrations of N of 0.1-0.5 mg/ml complete inactivation appears even at 2500 atm.

For example, measurement of the activity of solutions of purified myosin with N concentration 0.5 mg/ml at various pressures gave the following results:

Pressure in atm	1	500	1000	1500	2000	2500
ATP-ase activity μg P/mg N	2520	1500	1490	1200	500	0

We can also see that full inactivation of myosin at N concentrations about 0.1 mg/ml kept in the refrigerator for ten days occurs even under pressures of 2000 atm.

Inactivation under pressure in all cases is accompanied by only slight cloudiness of the solution of myosin. It is probable that the cloudiness was partly connected with denaturation of the more unstable muscle proteins of the type of myofibrillar globulins [6], apparently present in very small amounts in ordinary myosin preparations. Denaturative changes of myosin under pressure are chiefly in the form of disappearance of enzymatic (ATP-ase) activity while the protein keeps its hyophilic properties (solubility in 0.6 M KCl, high viscosity).

We also carried out experiments to study the effect of pH on stability of ATP-ase myosin toward high pressures. For the preparation of myosin solutions with different pH values we used borate buffer mixtures. Half the prepared protein solutions were submitted to the action of pressure, the other half served as controls. The experiments were carried out under pressure of 1500 atm; the pH of the solutions varied from 5.8 to 9.0.

The greatest decrease in enzymatic activity under pressure as compared with the controls was found in the range pH 6-7. At pH values above this the stability of the myosin ATP-ase to pressure increased and at pH about 8 was observed the greatest retention of enzyme activity of the myosin. Thus, for example, for myosin solutions which contained 0.12 mg N/ml at pH 6.5 the ATP-ase activity (μg P/mg N) after the action of pressure of 1500 atm fell from 890 to 490; at pH 7.8 ATP-ase activity fell from 950 only to 930. Probably this was related to the presence in the myosin of myosin with optimum ATP-ase activity on the alkaline side.

Change in Viscosity of Actin Solutions

The viscosity of solutions of activated actin under the influence of pressures of 1000-4000 atm is decreased; probably this is related to depolymerization of the actin. Thus, the viscosity of actin solutions which contain 1.14 mg N/ml after the action of a pressure of 1000 atm falls 2.6 times, and at a pressure of 4000 atm, 4 times.

Change in ATP-ase Activity of Actomyosin Preparations

The ATP-ase activity of actomyosin solutions (Fig. 1) is fully retained at pressures of 1000 atm; at 1500 atm it falls by about 30%. Complete inactivation of ATP-ase

in actomyosin is usually found at pressures of 2500-3000 atm. At a pressure of 500 atm there occurs a slight rise in the ATP-ase activity of actomyosin.

Change in ATP-ase Activity of Myosin- and Actomyosin with Time

In this series of experiments we used a pressure of 1500 atm. The protein solution remained under pressure for time intervals from 5 to 120 min.

Freshly prepared myosin solutions retained part of their enzyme activity (Fig. 2, curve 1) under the action of 1500 atm pressure for 80 and even 120 min. Solutions of myosin isolated a week or more before the experiment were completely inactivated even after 40-50 min (Fig. 2, curve 2).

The enzyme activity of actomyosin (Fig. 2, curve 3) under the same conditions (at 1500 atm) fell only slightly with time.

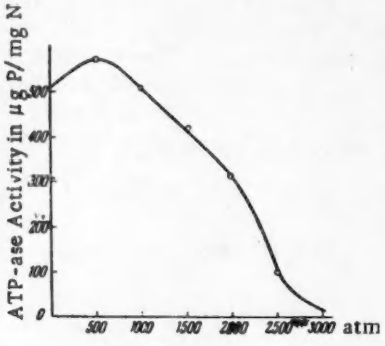


Fig. 1. Graph of effect of pressure on ATP-ase activity of actomyosin. Concentration of solution of actomyosin 0.57 mg N/ml

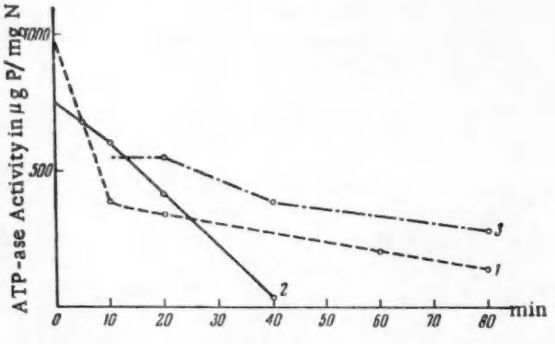


Fig. 2. Effect of length of action of a pressure of 1500 atm on ATP-ase activity of myosin and actomyosin. 1) Freshly prepared myosin; 2) myosin after 7 days keeping at 1-3°; 3) actomyosin. Concentration – 0.59 mg N/ml

Change of Viscosity of Actomyosin Solutions

In distinction from the ATP-ase activity, the relative viscosity (η) of actomyosin solutions which contained 0.5-0.9 mg N/ml fell sharply even under the influence of pressure of 1000 atm (Fig. 3), which may be related to a partial splitting of the actomyosin complex into myosin and depolymerized actin. With further increase in pressure, η rises somewhat. The latter effect may be explained as follows: as already mentioned, with rise in pressure, η of myosin solutions rises, and for actin solutions, it falls. Since actin in the actomyosin complex is present in smaller amounts and the fall in its viscosity under pressure is relatively slight, then η for the protein after maximum decrease at about 1000 atm (as a result of more or less full splitting of actomyosin into its components) can then gradually increase with further rise in pressure as a result of accumulation of

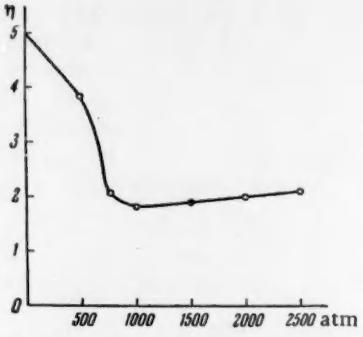


Fig. 3. Effect of pressure on relative viscosity of actomyosin. Concentration of actomyosin solution 0.57 mg N/ml.

denatured myosin. We studied the effect of ATP on the viscosity of actomyosin submitted to the action of high pressures (Table).

It follows from the data which we have given that actomyosin solutions which contain 0.5-0.9 mg N/ml even at pressures of 1000 atm almost fully lose the ability for decreased viscosity when ATP is added, which is connected with partial depolymerization of actin and splitting of the actomyosin complex into its components. However, ATP-ase activity of the myosin component of actomyosin is completely retained at a pressure of 1000 atm.

As to the effect of ATP on the relative viscosity of "artificial" actomyosin, prepared by mixing three volumes of myosin solution (containing 0.70 mg N/ml) with one volume of actin solution (containing 0.72 mg N/ml), it was unexpectedly found that such an "artificial" actomyosin shows somewhat greater stability with respect to high pressures. Thus, in "artificial" actomyosin, in distinction from "natural" actomyosin at equal concentrations of the proteins in solution after the action of

Effect of ATP on Relative Viscosity of Actomyosin Solutions after the Action of High Pressure. Actomyosin with Concentration 0.57 mg N ml. Added: 0.1 ml of 0.5% ATP per 2 ml of Protein Solution

Pressure atm	ATP-ase activity in μgP/mg N after 5 min	Relative viscosity		Decreased viscosity in % of original
		before adding ATP	after adding ATP	
1	512	2,52	1,62	35,7
1000	506	1,60	1,53	4,4
1500	456	1,50	1,45	3,4
2000	351	1,57	1,56	0
2500	105	1,74	1,70	4,0
3000	70	1,73	1,70	4,0
3500	35*	1,76	1,69	4,0

*The slight retention of ATP-ase activity is probably connected with presence in the preparation of admixed water soluble ATP-ase.

1000 atm pressure, there was a partial retention of the ability to decrease η in the presence of ATP.

The retention of this ability was the more definite, the higher the concentration of protein in the solution. However, at 3500 atm and probably also at lower values of the pressure, the "artificial" actomyosin also fully lost its ability to react with ATP. Thus, "natural" and "artificial" actomyosins do not show exactly the same degree of stability toward the action of high pressures. The reason for this effect still remains unclear.

SUMMARY

In this work we have studied the effect of high pressures (in the range from 500 to 4000 atm) on ATP-ase activity and viscosity of solutions of myosin and actomyosin and the viscosity of actin solutions. We have established that actin is distinguished by high sensitivity to the action of pressure; it undergoes depolymerization even under pressure of 1000 atm.

We have studied the effect of ATP on the viscosity of actomyosin submitted to the action of high pressure. We have established the absence of a parallel between loss of actomyosin ATP-ase activity and ability to decrease the viscosity in the presence of ATP. Evidently this fact is explained by the depolymerization of actin and splitting of the actomyosin complex into its components at pressures which do not cause a sharp decrease in the ATP-ase activity of the myosin component.

We have studied the effect of pH and length of action of pressure on ATP-ase of myosin and actomyosin.

We have established that under the influence of high pressure, the molecular weight, like the viscosity of myosin solutions, rises sharply.

We have suggested a modification of myosin which under the influence of high pressure has lost ATP-ase activity and ability to combine with actin, but which has retained its hydrophilic properties and solubility in 0.6 M KCl; we have called this baromyosin.

In conclusion we express thanks to Professor S.E. Bresler, and to Chief Scientific Assistant of the Institute of High Molecular Compounds, Academy of Sciences, USSR, S.Ya. Frenkel' for determining the sedimentation constant of myosin by the ultracentrifuge method. We express our gratitude to Docent E.V. Frisman for valuable advice in carrying out the work and to Chief Scientific Assistant S.E. Tukachinskii and Aspirant L. Shchagina for determining the molecular weight of myosin by the light-scattering method.

LITERATURE CITED

[1] I.I. Ivanov and T.I. Ivanova, Doklady Akad. Nauk SSSR 77, 657 (1951).

[2] I.I. Ivanov, E.A. Parshina, and N.I. Mirovich, Biokhimiya 24, 248 (1959).†

[3] F. Straub, Studies from the Institute of Medical Chemistry. Univer.Czg. 2, 3 (1942); 3,23 (1943).

[4] A. Szent-Gyorgyi, Muscular Activity [Russian translation] (Medgiz, Moscow, 1947).

[5] S.E. Bresler, Uspekhi Biol. Khimii 2, 66 (1954).

[6] I.I. Ivanov, Z.N. Zhakhova, I.P. Zinov'eva, et al., Biokhimiya 24, 451 (1959).†

[7] V.A. Engel'gardt and M.N. Lyubimova, Biokhimiya 7, 205 (1942).

[8] B.F. Poglazov, V. Bilushi, and A.A. Baev, Biokhimiya 23, 269 (1958).†

[9] B.F. Poglazov, Dissertation: Study of the Adenosine Triphosphate of Muscle and Some Plants (Moscow, 1957).

† Original Russian pagination. See C.B. translation.

CHANGES DURING STORAGE IN THE CHEMICAL COMPOSITION OF A VARIANT OF BRUCELLA OBTAINED UNDER THE INFLUENCE OF PHAGE

I. I. Dubrovskaya and N. N. Ostrovskaya

Division of Biochemistry and Brucellosis Laboratory,
Institute of Epidemiology and Microbiology,
Academy of Medical Sciences, Moscow
Translated from Biokhimiya Vol. 25, No. 3, pp. 511-516,
May-June, 1960
Original article submitted September 2, 1959

In the variability of microorganisms bacteriophage plays an important part along with changes in the external medium and the conditions of existence of the microbes. After its action, cells often assume new morphological, biological, and specific properties which may further be fully or partly fixed in the heredity (transduction phenomenon). Until recently it was considered that the chief factor in these transformations was nucleic acid. However, according to recent data, the important significance of proteins is not excluded [1].

What the mechanism of action of the various compounds is, and in what form they affect the enzyme systems of the bacterial cells, still remains unsettled.

One of the ways in which light can be cast on the answer to these interesting and important questions is to study the changes in chemical composition of the microorganisms after the phage has acted upon them.

Earlier we published the results of a study of the chemical composition of a culture of one of the variants of Brucella abortus 146 (variant 4), obtained under the influence of a phage of the "abortus" type [2]. In distinction from the original strain, the altered microbial cells of the brucella were covered with a mucous capsule, had practically lost virulent properties, and were not agglutinated by specific antisera. It was also shown that not all of the newly acquired characteristics were stable.

After long keeping of the variant on agar in a sealed tube at low temperature (2-4°) the brucella cultures altered by phage produced considerably less mucous layer and gradually regained their agglutinating properties, but remained weakly virulent.

The present communication concerns further changes found in the chemical composition of this variant.

METHODS

Depending on the period of keeping the variant, we grew and studied six different sets of cultures. The microbe population obtained by seeding the variant after keeping it from a day to four months was covered with an abundant mucous layer and did not have specific agglutinating properties. The cultures obtained after five and especially after six-month keeping of the variant formed considerably less mucus and gave a weak agglutination reaction. The final culture, grown after an eight-month keeping of the variant had visually lost the mucous capsule and had regained specific agglutinable properties.

To obtain the microbe mass, we grew the brucella on liver agar for three days after which the culture was mixed with physiological saline. Then the microbe mass was defatted by repeated treatment with acetone and ether.

From the acetone and ether extracts, after removal of the solvent by distillation in a stream of carbon dioxide, we obtained the lipids. By solution in chloroform and fractional precipitation with acetone they were separated into fractions of neutral fats, steroids, and a phosphatide fraction.

The content of free lipids of the brucella altered by phage was considerably increased compared to the initial culture and gradually fell as the variant was kept (Table 1). Thus, in cultures grown after six- and eight-month keeping, the content of free lipids reached the normal level. However, under these conditions there was a tendency to an increased fraction of neutral fats and steroids which appeared especially in cultures of the variant obtained after two months keeping.

The lowered content of free lipids agrees with the restoration of specific agglutinating properties in the culture.

It was earlier shown that cultures of the variant after growing on agar could be washed off and washed with physiological saline. The separate portions of physiological saline were combined, the microbe bodies were filtered off through a membrane filter and the solution was submitted to prolonged dialysis, concentrated, and then fractionated by precipitation with acetone. Two fractions were isolated: Fraction I was the substance which precipitated in 60% acetone, and fraction II was the substance which remained in solution at this concentration of acetone. The insoluble part was separated by centrifuging and dried with acetone and ether in a

TABLE 1. Content of Lipids and Their Fractions in Brucella Cultures Altered by Phage Depending on Period of Keeping the Variant

Microbe mass	Period of keeping the variant before seeding	Total lipid content in % on dry wt. of microbes	Neutral fats and steroids	Phosphatides
			in % on lipids	
Br. abortus 146 (initial culture) variant 4		9.77	74.88	22.52
	Immediately after formation	12.35	83.29	16.71
The same	2 months	13.10	85.17	13.46
" "	4 "	11.83	81.72	17.05
" "	5 "	11.21	80.02	18.04
" "	6 "	9.98	80.41	18.28
" "	8 "	9.38	80.15	18.52

vacuum desiccator. The soluble fraction, after removal of the acetone in a vacuum and dialysis, was dried lyophilically.

The specific properties of the fractions were studied by the method of double diffusion in agar according to Ouchterlony [3] with antisera obtained after immunization of rabbits by brucella altered by phage. The antisera were diluted with equal volumes of physiological saline.

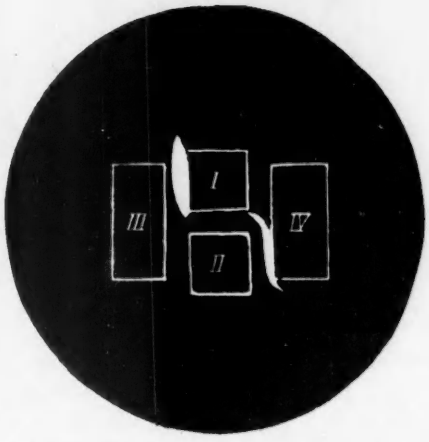


Fig. 1. Experimental precipitation in agar I) Fraction from physiological saline, precipitated by acetone, at concentration 10 mg/ml; II) fraction from physiological saline, soluble in 60% acetone, concentration 10 mg/ml; III) rabbit serum after immunization by a normal strain of Brucella abortus 146, titer 1:3200; IV) rabbit serum after immunization by a culture of variant 4, titer 1:3200. Adsorbed by fraction I.

Figure 1 shows that fraction I, precipitated from physiological saline by acetone, gives a clear line of precipitation with normal antibrucellosis serum, while the antiserum to the variant adsorbed by this fraction gives a positive reaction only with fraction II. Hence the antiserum to the variant contains an individual antibody which gives a positive precipitation reaction with substances which are not precipitated from physiological saline by acetone.

The yield of the fractions and their chemical characteristics are shown in Table 2. The content of reducing substances was determined by the Hagedorn-Jensen method, nitrogen by micro-Kjeldahl, phosphorus according to Fiske-Subbarow, RNA and DNA according to Schmidt and Thanhauser, hexosamine according to Elson and Morgan. Protein was calculated from total nitrogen after allowing for the N of nucleic acids and the N of hexosamine by multiplying by the coefficient 6.25.

We note the sharp decrease in content of substances soluble in physiological saline in the cultures obtained after keeping the variant for five months. Beginning from just this period in the culture of the variant we find a restoration of the specific agglutination properties.

Comparison of different preparations of fractions I and II isolated in different sets of cultures of the variant shows us the existence of chemical differences. Some variations chiefly in the content of phosphorus, nucleic acids, and hexosamine may be explained by the fact that neither fraction I or fraction II is a definite chemical compound, but a mixture of various organic substances whose relative content is related to the individual peculiarities of the microbe population in each set of cultures.

The fraction of substances soluble in 60% acetone contained, along with polysaccharides and hexosamine,

TABLE 2. Yield and Chemical Characteristicsof Fractions Isolated from Physiological Saline

Period of keeping variant 4 before seeding	Yield in % on microbe mass	Reducing substances	Nitrogen	Phosphorus	RNA	DNA	Protein	Hexosamine
		in % absolute dry weight of fraction						
Fraction precipitated by acetone								
Immediately after formation	55	26,87	6,51	1,13	4,88	0,51	34,68	4,06
2 months	57	26,24	6,30	1,62	5,06	0,76	32,56	4,50
4	50	24,31	7,13	1,69	3,98	0,56	38,18	3,96
5	28	25,54	6,38	1,27	3,68	0,81	35,44	3,3
6	14	24,81	7,03	1,30	4,06	0,48	38,81	3,48
8	11	22,12	7,15	1,05	2,76	0,32	41,25	3,15
Fraction which remains in solution								
Immediately after formation	117	18,47	10,91	0,27	2,35	0,0	63,19	7,14
2 months	101	17,82	10,75	0,65	2,82	0,0	61,56	8,13
4	123	15,21	10,65	0,42	2,65	0,0	61,94	6,12
5	46	18,76	10,63	0,37	2,12	0,0	62,0	6,43
6	18	15,63	10,93	0,53	2,18	0,0	63,63	6,81
8	16	14,03	11,07	0,38	1,80	0,0	65,0	6,13

Fig. 2. Carbohydrate composition of the fraction isolated from the wash liquid of the variant culture A) Polysaccharide fraction of substances precipitated by acetone; B) polysaccharide fraction of substances which remain in solution. 1) Uronic acid; 2) galactose; 3) glucose; 4) mannose; 5) arabinose; 6) xylose; 7) ribose; 8) rhamnose; 9) deoxymethylpentose

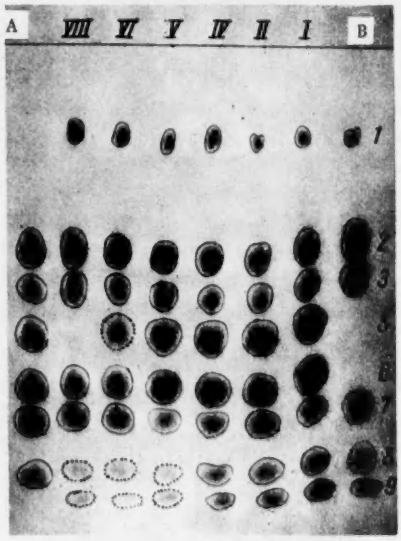


Fig. 3. Carbohydrate composition of microbe bodies, variant 4, grown after different periods of keeping. A) Standard B) initial culture; I-VIII, cultures of variant 4 kept 1,2,4,5,6,8 months respectively. 1) Uronic acid; 2) galactose; 3) glucose; 5) arabinose; 6) xylose; 7) ribose; 8) rhamnose; 9) deoxymethylpentose.

a considerable amount of protein substances and ribonucleic acid. The separate fractions isolated from physiological saline also differed in qualitative composition of the polysaccharides (Fig. 3).

Serological study of the fractions isolated from physiological saline, chromatographic determination of the composition of their polysaccharides, and also the data of total chemical analysis permit us to consider that fraction I contains chiefly lysed microbe bodies, while fraction II consists chiefly of substances of the mucous capsule. In cultures obtained after six and eight months keeping of the variant, in spite of the absence of a visible capsule, as a result of serological and chemical analysis, there is still some quantity of capsule substance.

The defatted microbe mass grown after various periods of keeping of the variant was studied for content of reducing substances, nitrogen, phosphorus, RNA, DNA, and hexosamine. The results of the analysis are given in Table 3.

The data presented show that in the variant as it is kept at 2-4° there occur enzymatic processes which lead to further shifts in chemical composition of the microbe

body. In cultures of the variant grown immediately after obtaining it there is a sharp increase in DNA content and a decreased content of RNA; when the cultures are grown after keeping from two to six months, the DNA content gradually decreases, but the RNA content increases. The protein content, if we do not consider the very small increase in the culture in the period from two-to five-month keeping of the variant, remains practically constant. There is a fall in content of hexosamine in cultures grown after six and eight months keeping of the cultures, a fall of almost half its content in the initial culture. Such a fall in content of amino sugars with partial or full loss of virulent properties by the microorganisms as compared to the acutely virulent strain is also encountered in work with other pathogenic microorganisms [4, 5]. We should mention that in our case the brucella cultures after the action of phage also almost lose their virulent properties.

The qualitative composition of the microbe body polysaccharides of the variants from different series were studied by paper chromatographic analysis. A sample of defatted material was hydrolyzed with 1 N H_2SO_4 on a boiling-water bath for 4-5 hours. After removal of the sulfuric acid by baryta, the hydrolysate was concentrated to small volume. Purification from high-molecular-weight compounds was carried out by addition of alcohol to a concentration of 80-90%. After centrifuging, the precipitate was washed several times with 90% alcohol and sucked off. The supernatant liquid and wash water were freed from alcohol and evaporated dry. The residue was dissolved in an amount of water suitable for deposition on the paper (calculated on 200 μg of reducing substance) and submitted to chromatographic separation. The solvents were butanol–acetic acid–water (4:1:5) and also butanol–pyridine–water–benzene (5:3:3:1). The paper was Leningrad slow, the developer, aniline phthalate. The results of the chromatographic separation are shown in Fig. 2.

In distinction from the initial brucella strain the culture altered by phage shows pentoses: arabinose and xylose. However, when the material is deposited in equal amounts (based on reducing substances) in cultures grown after keeping the variant for six months, the arabinose spot is markedly weakened (No. 5) and in the culture obtained after keeping for eight months (No. 6), arabinose is absent.

In cultures of the variant grown after keeping for various periods, there is also a marked change in the content of the rapidly moving sugars rhamnose and deoxymethylpentose. The chromatograms show that in cultures of the variant after short keeping the spots of these sugars are greatly intensified. In the case of cultures grown after five or more months keeping, the quickly moving sugars are found as traces.

The composition of the polysaccharide fraction substances isolated from the wash liquids of the cultures

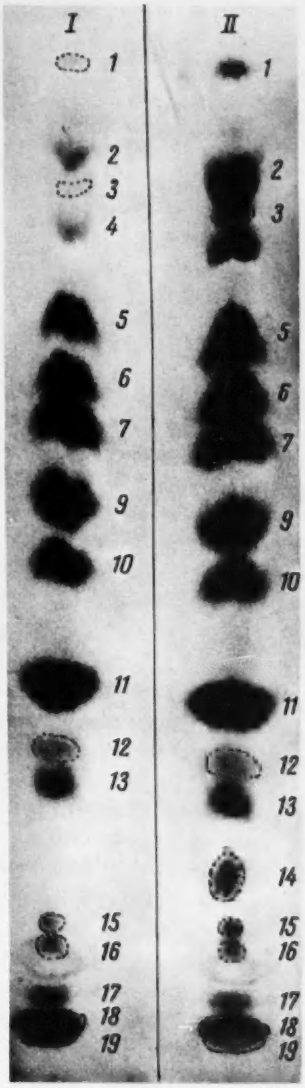


Fig. 4. Amino acid composition of the proteins of the variant and initial cultures of brucella. I) Microbe mass of variant (series VI); II) microbe mass of initial culture 1) Cystine; 2) lysine; 3) histidine; 4) arginine; 5) aspartic acid; 6) serine; 7) glycine; 9) glutamic acid; 10) threonine; 11) alanine; 12) proline; 13) tyrosine; 14) α-aminobutyric acid; 15) methionine; 16) valine; 17) phenylalanine; 18) isoleucine; 19) leucine. Paper---Leningrad slow.

TABLE 3. Chemical Characteristics of Microbe Bodies of the Variant Grown after Various Periods of Keeping

Microbe mass	Period of keeping variant before seeding	Reducing substances	Nitrogen	Phosphorus	RNA	DNA	Total nucleic acids	Protein	Hexosamine
Initial culture of Br. abortus 146 Variant 4	Immediately after formation	9,82	12,20	1,82	9,22	3,82	13,22	69,63	3,40
The same	2 months	9,27	12,16	2,24	6,58	7,14	13,72	68,75	3,05
"	4 "	8,91	12,54	2,08	7,10	6,42	13,52	72,0	2,74
"	5 "	8,98	12,36	2,04	7,35	6,15	13,50	71,0	2,58
"	6 "	7,78	12,94	1,65	6,92	5,03	12,95	74,65	2,12
"	8 "	9,52	12,01	1,85	9,31	4,32	13,65	68,94	1,85
		8,71	12,10	1,87	10,20	4,30	14,50	69,06	1,80

altered by phage is the same for all series (Fig. 3). The fraction precipitated by acetone contains uronic acid, galactose, glucose, arabinose, xylose, ribose, rhamnose, and deoxymethylpentose. The fraction which remains in solution contains uronic acid, galactose, glucose, mannose, ribose, rhamnose, and deoxymethylpentose.

The microbe body proteins of the variant grown after various periods of keeping were also studied by the chromatographic method. For this purpose, the hydrolysate of the defatted microbe mass (6 N HCl for 24 hours), after removal of humin, hydrochloric acid, and salts, was deposited on paper in an amount corresponding to 60 μg of protein N. Separation of the amino acids was carried out with two solvents: butanol—acetic acid—water (40:10:50) and the same ingredients in the ratio 40:15:5, passing each of them three times with successive drying of the paper.

The qualitative composition of the microbe body proteins of the variant remained the same, independent of the period of keeping it. The protein of the variant cells in all six sets of experiments differed from the protein of the initial culture by the absence of α-amino butyric acid, and, beginning chiefly from the fourth series (five months keeping), by the lower content of cystine, lysine, and arginine (Fig. 4).

It follows from the above material that the change in specific properties with almost complete loss of virulence in the virulent culture of Brucella abortus 146, which originates under the action of phage, is the result of severe damage to the metabolism which is also shown in changes in the external form of the bacterial cells and colony. Their form and size change, and the cells are covered with mucus. The latter effect is characteristic not only for the action of phage, but is evidently also a protective reaction of many microorganisms when their important vital functions are damaged. Thus, the formation of mucus is often found in pathogenic bacteria in the action of antibiotics [6], under the influence of ionizing radiation [7], and of mechanical injury [8].

Destruction of normal life activity of brucella, connected probably with blocking or inactivation of a number of enzyme systems, produces a considerable shift in the chemical composition of the microbe body; the increased content of free lipids, change in content of DNA and RNA and hexosamine, and change in carbohydrate composition of the polysaccharides (appearance of arabinose and xylose) and the amino acid composition of the proteins (absence of α-aminobutyric acid).

However, in view of the plasticity of the bacterial body, the ability of its metabolism to adapt quickly to different conditions of existence, new enzyme systems can be gradually synthesized in the cells to replace those lost. Such adaptation perhaps occurs during keeping of brucella altered by phage in the process of reciprocal adaptation of the metabolisms of microorganism and phage. It is still possible that chemical changes in the cultures of the variant grown after various periods of keeping it reflect these phenomena to a definite extent.

The shift which we have observed in chemical composition of the cultures of variants with different lengths of keeping (change of carbohydrate composition of the polysaccharides, approach to normal content of free lipids and to the usual ratio of nucleic acids for the original strain of brucella) is accompanied by morphological changes (gradual decrease in mucous covering, restoration of the original form of the cells and colony), and also by restoration of the specific agglutinating properties.

SUMMARY

Cultures of Brucella abortus 146 (variant 4) under the influence of phage under conditions of prolonged keeping regain some of the properties which were lost.

At the basis of this effect lies a shift in biochemical processes expressed (with the limits of our investigation) by a change in carbohydrate composition of the polysaccharides and also the content of nucleic acids and free lipids, as a result of which such cultures approach

the behavior of normal brucella cells which have not been affected by phage.

The hexosamine content in the cultures of the variant grown after a long period of keeping is decreased.

Qualitative changes in the protein content of the microbial body of the variant grown after various periods of keeping were not observed.

LITERATURE CITED

[1] L. Levene, J. Berlow, and H. Vankins, Federat. Proc. 17, 522 (1958).

[2] I.I. Dubrovskaya, N.N. Ostrovskaya, and A.I. Glubokina, Biokhimiya 23, 523 (1958).*

[3] O. Ouchterlony, Prog. in Allergy 5, 1 (1958).

[4] V.D. Timakov, D.G. Kudlai, V.G. Petrovskaya, A.M. Korneeva, and S.A. Bogatyreva, Zhur. Mikrobiol. Epidemiol. Immunobiol. 2, 23 (1959).

[5] E. Mikulyashek and Ya. Dzhulynskaya, Biol. Polsk. Akad. Nauk 10, 21 (1956).

[6] A.F. Moroz and O.M. Kostyukovskaya, Variability of Microorganisms 2 [in Russian] (Izd. AMN SSSR, Moscow, 1957), p. 65.

[7] Z. G. Pershina, S. G. Komm, and N. N. Solov'ev (Izd. AMN SSSR, Moscow, 1957).

[8] E. I. Koptelova, (Izd. AMN SSSR, Moscow, 1957).

*Original Russian pagination. See C. B. translation.

NUCLEOPROTEIN FRACTIONS OF VACCINE STRAINS OF PLAGUE BACTERIA

M.L. Bekker, A.Z. Kutsemakina, and É.A. Chernova

Antiplague Research Institute of the Caucasus and Transcaucasus, Stavropol

Translated from Biokhimiya Vol. 25, No. 3, pp. 517-522,
May-June, 1960
Original article submitted September 4, 1959

The nucleoproteins of plague bacteria were obtained as early as 1897 by Lustig and Galeotti [1]. Recently similar preparations have been obtained several times [2, 3]. However, the study of these substances was limited to the determination of their biological properties in connection with the problem of obtaining a useful preparation for immunization against the plague. In recent times, in connection with the work of Backer et al. [4], which showed that the immunologically active fraction of plague bacteria did not contain nucleic acids (NA), interest in studying the nucleoteins of these organisms lessened considerably. There is only fragmentary information as to the NA content in the cells of plague bacteria [5].

It should be mentioned, however, that the study of bacterial nucleoproteins in connection with the problem of variability of the organisms gave very interesting results [6, 7] and should be continued with objects of bacterial origin. There is some interest from our point of view in the problem of the NA content and fractional composition of the nucleoproteins of plague bacteria in connection with the conditions of their growth.

In the present communication we give data on the NA content and composition of the nucleoprotein fraction of two vaccine strains of plague bacteria in growth with aeration.

MATERIALS AND METHODS

Strains. We studied avirulent strains of plague bacteria, type EB and No. 1, both strains belonging to the oceanic type (not decomposing glycerol).

Nutrient medium. For growth we used the usual medium used in production on pancreatically digested meat with an amino nitrogen content of 0.1-0.15%. Growth with aeration was carried out under semi-works conditions in a reactor with a capacity of 45 liters connected with a bubbler. Air was supplied in the amount of 0.7-1 volume per volume of nitritive medium per minute. The temperature was automatically maintained at 37°.

Chemical investigation. The microbe body was separated from the medium by centrifuging, washed with isotonic NaCl solution, and suspended in alcohol, then washed three times with alcohol, three times with ether, and dried. The content of residual moisture varied from 3 to 10% (Table 1).

In the dry bacterial mass we determined the content of total N by the micro-Kjeldahl method, NA spectrophotometrically according to Webb [8], DNA with diphenylamine, and RNA by the orcinol method. The total NA content and the sum of DNA and RNA determined separately agree well. The content of protein and NA was calculated on the basis of these determinations.

Fractionation of the dry bacterial mass was carried out by the method of Mirsky and Pollister in the modification of Belozerskii [9]. The fraction obtained from the extract with 0.14% NaCl was designated by A, that from 1 M salt solution by B, and that from 0.2% alkali extract by C. The numbers 1, 2, 3 denote fractions precipitated from the corresponding extracts by acetic acid, alcohol, and acetone (for example, the fraction precipitated from 0.14 M salt extract by acetone is designated as fraction A_3, etc.).

RESULTS

In Table 1 we give the data for determination of content of protein and NA in the bacterial mass obtained under different conditions of growth. As the table shows, the variation in content of total N does not have a regular character and evidently does not depend on time of growth and addition of carbohydrates to the medium.

The content of NA was somewhat higher in the bacterial mass of 24-hour growth than in cultures grown for longer times. It was considerably increased by addition of carbohydrates to the mixture. The biomass then increased with unit volume of the medium. This was shown especially clearly in those experiments in which we artificially created conditions for the best utilization of carbohydrates, keeping the pH at the optimum for the growth of the cultures (experiments 15 and 18).

The change in content of DNA was most marked. Thus, in the 24-hour culture of strain EB the content of DNA was more than double as compared to the 48-hour culture of the same strain. The difference in DNA content in strain No. 1 was also sufficiently great.

TABLE 1. Content of Total Nitrogen and NA in the Bacterial Mass of Plague Bacteria
We give the average figures from these experiments:

No. of bacterial mass	Strain	Time of growth, hours	Yield of biomass fr. 1 liter of medium, g	Moisture, %	Content in % absolute dry weight					
					$N_{tot.}$	Protein + NA	NA	DNA	RNA	protein
4	EB	24	0,194	7,8	16,23	100,1	17,9	3,86	14,04	82,2
5	"	24	0,125	5,6	14,19	88,6	14,55	3,53	11,02	74,05
6	"	48	0,140	8,1	14,48	90,5	14,42	1,54	12,88	76,08
7	"	48	0,170	7,5	13,61	85,2	10,8	1,27	8,53	74,4
8	No. 1	24	0,166	4,6	13,95	87,2	15,8	2,1	13,7	71,4
9	"	24	0,183	12,2	15,02	93,9	18,21	2,85	15,36	75,69
10	"	48	0,128	2,8	14,71	92,0	14,48	1,73	12,75	77,52
11	"	18	0,121	4,6	13,4	83,7	17,6	1,75	15,85	66,1
12	"	48	0,144	3,5	14,51	90,8	14,5	1,74	12,76	76,3
13	"	24	0,193	7,2	13,4	83,7	20,64	2,96	17,6	63,06
15	"	24	0,325	3,6	11,76	75,5	19,3	3,34	16,1	56,2
18	"	24	0,286	4,8	15,1	94,4	17,0	3,74	13,23	77,38

Note: Bacterial masses 4-12 were obtained from media without addition of carbohydrates; bacterial masses 13, 15, and 18 from media with such addition during growth. In experiments 15 and 18 the pH during growth was kept within the limits 6.8-7.2.

Under conditions of insufficient carbohydrate, the percent content of DNA in the bacterial mass of strain No. 1 was lower than in strain EB, but when carbohydrate was added to the medium, and especially when the pH of the medium was kept between 6.8 and 7.2, the DNA content was the same as in the bacterial mass of strain EB of the corresponding time of growth.

Thus, the addition of carbohydrates during growth of the plague bacteria under aeration conditions leads to increased content of NA and appreciably increases the yield of biomass.

Fractionation of the plague bacteria gave a very high yield of total nitrogen and NA. In all the experiments the yield exceeded 70% and in many of the experiments reached 80-90% of the total amount of substance used for fractionation. In this respect plague bacteria differ greatly from some other gram-negative bacteria. Thus, in the fractionation of dysentery bacteria we [10] obtained only 28-30% of the material used for the fractionation. Similar results were obtained by Kudlai et al. [11] in fractionation of the proteins of the alkali-forming variant of bacteria of the intestinal group. The yield in our experiments with plague bacteria was evidently as high as the method permitted, considering the numerous extractions and reprecipitations. We can assume that such a high yield of nitrogenous products is explained by the low activity of the proteases in this type of bacteria.

The data on distribution of total nitrogen and NA by fractions is characterized by considerable variety (Table 2). About 20-40% of the total nitrogen is not removed by our extracting agents and makes up the so-called residue (X). The remaining 60-80% of these substances are distributed between the different fractions. About half of this amount was found in the fractions extracted by salt, especially in the 0.14 M salt extract. An exception was the 24-hour bacterial mass of strain EB, where the greater part of the total N and the NA were found in the fraction from the 1 M NaCl extract.

More than ninety percent of the nucleic acids were removed from the cells by solutions of NaCl and NaOH. The greater part of the RNA was found in the 0.14 M salt extract and was precipitated by acidification of the latter with alcohol (fractions A_1 and A_2). DNA was found chiefly in the fractions from the extracts with 0.2% NaOH. As Table 2 shows, ther is no difference in distribution of total nitrogen and NA in fractions from bacteria grown on media with added carbohydrates and without them.

It is interesting to observe that the distribution of NA by fractions from the 0.14 M salt extract (Table 3) differs in strains EB and No. 1 very considerably. In the first, with acificiation of the extract, only about one third of the total nitrogen and RNA and about one half of all the DNA enter the precipitate, while in the corresponding fraction from strain No. 1, more than 2/3 of the total nitrogen and RNA and almost all the DNA precipitate. Here the age difference is less than the difference between the strains. This change also depends little on the addition of carbohydrates to the medium.

In Table 4 we give values for the ratio of total nitrogen to NA phosphorus.

Table 4 shows that in most of the fractions obtained from the bacterial mass by extraction of the latter with salt solutions, the value of the ratio N/P is less, that is, the relative amount of NA in them is greater, than in the original bacterial mass. In fraction C the value of the ratio is also sufficiently low. These data indicate that fractions obtained from these extracts are nucleoproteins with a rather high NA content. In special cases the value of the ratio is markedly lower than occurs in

TABLE 2. Distribution of Total Nitrogen and NA among the Fractions in Plague Bacteria
We give the average figures from these experiments:

Fraction	N_{total}					RNA					DNA				
	EB* 24 hr. 4**; 5	EB 48 hr. 6; 7	№ 1 24 hr. 8; 9	№ 1 48 hr. 10; 12	№ 1 24 hr. 13; 15; 18	EB 24 hr. 4; 5	EB 48 hr. 6; 7	№ 1 24 hr. 8; 9	№ 1 48 hr. 10; 12	№ 1 24 hr. 13**; 15; 18	EB 24 hr. 4; 5	EB 48 hr. 6; 7	№ 1 24 hr. 8; 9	№ 1 48 hr. 10; 12	№ 1 24 hr. 13; 15; 18
A_{1-3}	29,1	18,0	23,6	34,2	28,9	83,6	33,2	60,6	63,5	69,8	19,4	35,0	16,1	12,6	9,0
B_{1-3}	32,0	1,5	2,2	2,3	5,1	10,1	1,5	8,4	4,2	14,7	2,4	0,0	4,7	4,0	4,4
$A+B$	61,1	19,5	25,8	36,5	34,0	93,7	34,7	69,0	67,7	84,5	21,8	35,0	20,8	16,6	13,4
B_{1-3}	21,0	45,0	31,6	29,8	39,5	2,6	61,0	25,6	29,4	14,5	73,5	63,7	61,3	74,7	85,3
$A+B+C$	82,1	64,5	57,4	66,3	73,5	96,3	95,7	94,6	97,1	99,0	95,3	98,7	82,1	91,3	98,7
X	18,9	35,5	42,6	33,7	26,5	3,7	4,3	5,4	2,9	1,0	4,7	1,3	17,9	8,7	1,3

*In this and later columns we show the strain, time of growth, and number of experiments.
**Bacterial masses 4-12 were obtained by growing the corresponding strains without addition of carbohydrates; bacterial masses 13-18 were grown on media with addition of carbohydrates.

TABLE 3. Distribution of Total Nitrogen and NA in Fractions from 0.14 M NaCl Salt Extract

Strain	No. of bacterial mass	Culture period, hrs	N_{total}		RNA		DNA	
			A_1	A_{2-3}	A_1	A_{2-3}	A_1	A_{2-3}
EB	4,5	24	37,5	62,5	32,5	67,8	39,3	60,7
EB	6,7	48	28,9	71,1	24,5	75,4	52,8	47,2
№ 1	8,9	24	68,7	31,3	64,1	35,9	97,8	2,2
№ 1	10, 12	48	85,3	14,7	93,4	6,6	100,0	0,0
№ 1	13, 15, 18	24	72,9	27,1	69,9	30,1	97,7	2,3

Note: A_1---fraction precipitated from 0.14 M salt extract on its acidification; A_{2-3} --- fractions precipitated by alcohol and acetone.

TABLE 4. Ratio of Total Nitrogen to Phosphorus of NA in the Bacterial Mass and Fractions from it.

Fraction	EB 24 hr. 4–5	EB 48 hr. 6–7	№ 1 24 hr. 8	№ 1 24 hr. 9	№ 1 48 hr. 10	№ 1 48 hr. 12	№ 1 24 hr. 13	№ 1 24 hr. 15	№ 1 24 hr. 18
BM	9,4	9,5	9,1	8,5	10,5	10,3	6,7	6,3	9,2
A_1	4,8	5,2	4,3	3,6	6,0	3,5	3,4	4,3	4,6
A_2	1,7	4,0	3,3	2,0	22,3	3,0	1,7	2,1	2,2
A_3	17,8	7,0	3,6	4,3	24,0	5,6	6,6	5,3	8,9
B_2	30,8	3,0	2,0	—	3,3	—	1,9	2,6	2,0
B_3	69,1	4,0	2,0	5,4	8,0	11,9	6,5	7,0	3,1
C_1	13,3	6,9	5,9	9,5	7,7	8,7	5,0	33,6(a)	73,4(a)
								9,6(b)	8,4(b)
C_2	7,7	3,7	4,4	56,0	—	107,0	—	—	—
C_3	10,0	—	1,5	9,5	12,4	—	96,2	44,1	62,5
X	50,0	88,8	68,4	39,5	40,0	41,0	89,9	306,0	624,0

Note: Where line is drawn, the NA content in the fraction was not determined; C_{1a} and C_{1b} are fractions obtained by separate precipitation of the alkaline extract.

natural nucleoproteins. In the residue (X) the NA content is slight.

It is clear from the above data that DNA is extracted from the plague bacterial mass especially by the alkali solution. We have tried to establish the dynamics of extraction of DNA. Usually the alkali extraction was repeated eight to nine times and the mixed extracts were submitted to fractionation. In experiments 15 and 18 the first four and the last four alkali extracts were fractionated separately. On this we found that the fraction precipitated by acetic acid from the first four alkali extracts contained 30-60% total nitrogen, and only 7-10% DNA was removed by this solvent. This was reflected in the value of the N/P ratio (Table 4) which for the first four extracts (C_{1a}) was 33.6 and 73.4, and for the last (C_{1b}), 9.6 and 8.4. Thus, DNA is very strongly fixed in the cell and enters the alkali solution only after repeated treatment, which is accompanied by complete destruction of all the cell structures.

DISCUSSION OF RESULTS

The decrease is content of nucleic acids which we observed in the process of intensive growing of plague bacteria resembles that in some other gram-negative bacteria [12] grown under analogous conditions. As to the fractional composition of these bacteria, here we can note some peculiarities. In our experiments, fractions obtained from alkali extracts contained considerably more NA than the analogous fractions in the experiments of other authors [13]. In particular, in these fractions most of the DNA extracted from the cells and a small part of the RNA could be found. DNA was extracted by alkali only by frequently repeated extraction (experiments 15 and 18) and evidently was strongly fixed in the cell.

The composition of the protein fraction of plague bacteria of the given strains was sufficiently constant and qualitative differences depending on time and some of the changes in conditions of growing (addition of carbohydrated during growth) could not be found. There was only a clear difference in the distribution of NA in the fractions in the two different strains of plague bacteria. This difference appeared chiefly in the fractions obtained from the 0.14 M salt extract. We can assume that this relation reflects to a definite degree a difference in character of complex-formation of the protein with NA, but for confirmation of this suggestion a special study is necessary, since our method of fixing the bacteria and the following treatment can lead to altered relations of what was in the living cell.

SUMMARY

We have studied the content of proteins and nucleic acids (NA) in vaccine strains (EB and No. 1) of plague bacteria grown with aeration, and in the nucleoprotein fraction obtained from dry bacterial mass of these bacteria, by the method of successive extractions with 0.14 M and 1 M NaCl and 0.2% NaOH followed by fractionation of the extracts.

We have established that in the process of growth of the bacteria the protein content does not change regularly and the NA content decrease.

With addition of carbohydrates to the medium, the NA content, and especially the DNA, increases during growth (as compared to that in bacteria grown in media without carbohydrates). At the same time the yield of the biomass in a unit volume of the medium also increases.

The fractions obtained from the dry bacterial mass of plague bacteria are nucleoproteins with a relatively high NA content. The residue after fractionation is protein with a relatively low NA content but in all the experiments some of the latter was found in it. RNA was extracted chiefly by 0.14 M salt solution and DNA was observed in the alkali extract, but in all the experiments some RNA accompanied the the main mass of DNA and about 15-20% of the latter entered the salt extracts.

The chief mass of DNA and the RNA which accompanied it were extracted only by frequently repeated treatment with alkali, which indicates the strong fixation of it in the cells of the plague bacteria. Qualitative differences in distribution of total nitrogen and NA in fractions from the same strain of bacteria were not observed. We noted only differences between strains EB and No. 1 in distribution of total nitrogen and NA in the fractions from 0.14 M salt extracts.

LITERATURE CITED

[1] G. Lustig and G. Galeotti, Dtsch. med. Wochenschr. 23, 289 (1897); Brit. Med. J. 1, 206 (1901).

[2] S. Rowland, J. Hyg. 10, 536, (1910); J. Hyg. 11, 11 (1911)

[3] M. Schutze, Brit. J. Exptl. Pathol. 13, 289, 293 (1932).

[4] E.E. Backer, H. Sommner, L.E. Foster, E. Meyer, and K.F. Meyer, J. Immunol. 68, 131 (1952).

[5] E.M. Gubarev and N.N. Ivanovskii, Biochemistry of Plague Bacteria [in Russian] (Medgiz, Moscow, 1958).

[6] A.N. Belozerskii, A.S. Spirin, L.G. Kudlai, and A.G. Skavronskaya, Biokhimiya 20, 656 (1955).

[7] V.D. Timakov, D.G. Kudlai, A.S. Spirin, and A.N. Belozerskii, Zhur. Mikrobiol., Epidemiol. Immunobiol. 8, 20 (1955).

[8] J.M. Webb, J. Biol. Chem. 230, 1023 (1958).

[9] A.N. Belozerskii and N.I. Proskuryakov, Practical Handbook of Plant Biochemistry [in Russian] (Sov. Nauk, Moscow, 1951).

[10] M.L. Bekker, Variability of Microbes 3 [in Russian] (Izd. AMN SSSR, Moscow) (in press).

[11] D.G. Kudlai, A.G. Skavronskaya, and G.Ya. Zhiznevskaya, Variability of Microbes 2 [in Russian] (Izd . AMN SSSR, Moscow, 1957), p. 186.

[12] M.L. Bekker, Trudy Khar'kovsk Inst. Vaktsin i Syvorotok 23, 149 (1956).

[13] D.G. Kudlai, A.G. Skavronskaya, and A.S. Spirin, Variability of Microbes 2 [in Russian] (Izd. AMN SSSR, Moscow, 1957), p. 202.

PENTOSE METABOLISM AND THE EFFECT OF ORTHOPHOSPHATE ON THE PATH OF DEGRADATION OF SUGARS IN STREPTOMYCES AUREOFACIENS*

Shên Sang-ch'ung and Ch'ang Yung-p'u
with participation of Ku T'i-ên

Laboratory of the Physiology of Microorganisms, Institute of Plant Physiology,
Chinese Academy of Sciences, Shanghai
Translated from Biokhimiya Vol. 25, No. 3, pp. 523-531,
May-June, 1960
Original article submitted September 8, 1959

The previous investigation [1] established that in the carbohydrate metabolism of the mycelia cells of Streptomyces aureofaciens there was participation both of the glycolytic cycle (Embden-Meyerhof-Parnas system) and the pentose cycle (hexose monophosphate bypass). In the present communication we report a further study which confirms the value of the pentose cycle in these actomycetes. The existence is shown of an interconnection between the two alternative paths for carbohydrate breakdown and the dynamic transfer from one to the other under the influence of orthophosphate.

MATERIALS AND METHODS OF STUDY

Streptomyces aureofaciens, strain A_3 was grown at pH 6.4 on a synthetic medium with the following composition: 2.5% glucose, 0.5% asparagine, 0.3% $MgSO_4$, 0.005% $ZnSO_4$, 1.5% KH_2PO_4. After 36 hours growth at 27° in a reciprocating rocker, the mycelia cells were separated by centrifuging and washed several times at 5° to remove traces of antibiotics retained on the surfaces of the cells. To the mycelia residue we added three times the volume of cold acetone and stirred the suspension for 10 min, after which the acetone treated cells were separated on a Buchner funnel and dried in a vacuum. A cell-free extract was prepared from the acetone-treated powder.

Sedoheptulose was determined by the method of Horecker [2] and Dische [3], hexoses by the aid of cysteine and H_2SO_4 (CYRIW method according to Dische [4]), fructose according to Roe [5], PGA according to Meyerhof and Lohmann by determination of alkali-labile phosphate [6].

F-1,6-P was determined by a spectrophotometric method of Slater [7]. The reaction mixture contained the experimental sample (or a standard solution of F-1,6-P), DPN-H, aldolase and glycerophosphate dehydrogenase in a tris-hydroxymethylaminomethane buffer at pH 7.6. Aldolase and glycerophosphate dehydrogenase were prepared according to Racker [8] from rabbit muscle. DPN was obtained by the method of Burton and Wilson [9] and was transformed into DPN-H by enzymatic reduction with yeast alcohol dehydrogenase.

The phosphate esters of the sugars were isolated from the mycelia cells by ion-exchange chromatography on a column with Dowex-1 resin (Cl^- form) according to Khym and Cohn [10]. The products of the transformation of R-5-P were separated by the method of Horecker [11]; the enzymatic reaction was stopped by addition of 5% TCA and the neutralized protein-free filtrate was submitted to chromatography on a column of Dowex-1 in the formate form.

RESULTS OF THE INVESTIGATION

The presence in the cell-free extract of enzyme systems of both alternative cycles of carbohydrate degradation was established in the previous work [1]. We have obtained further data which confirm that in the intact, living mycelia both systems act. The mycelia collected after 36hours growth and washing were ground for 20 min in a mortar with three volumes of distilled water and one volume of glass powder (Pyrex). The extract separated by centrifuging was kept for three min on a boiling water bath and on cooling was brought to a pH of 3.5; it was centrifuged and the pro-

* Abbreviations used in this paper: HDP) hexose diphosphate; HMP) hexose monophosphate cycle; G-1-P) glucose-1-phosphate; G-6-P) glucose-6-phosphate; DPN) diphosphopyridine nucleotide; Xu-5-P) xylulose-5-phosphate; R-5-P) ribose-5-phosphate; Ru-5-P) ribulose-5-phosphate; S-7-P) sedoheptulose-7-phosphate; PGA) 3-phosphoglyceraldehyde; 6-P-G) 6-phosphogluconate; PDA) phosphodihidroxyacetone; F-1-P) fructose-1-phosphate; F-6-P) fructose-6-phosphate; F-1,6-P) fructose-1,6-diphosphate; EMP) Embden-Meyerhof-Parnas system; E-4-P) erythrose-4-phosphate.

tein precipitate was discarded. The supernatant liquid was brought to pH 8.5 and chromatogtaphed on an anionite to separate and identify the intermediate phosphorylated products of the EMP and HMP systems. We were especially interested in the presence in the mycelia cells of G-1-P. The fraction eluted immediately after F-6-P, in its behavior in color reactions with orcinol and the absorption spectrum of the product of the orcinol reaction, was similar to the pentose phosphates. However, in the chromatographic separation this fraction was distinct from pentose phosphate esters and required further identification (Fig. 1).

Pentose metabolism. It was showed in the previous work that on oxidation of G-6-P and 6-P-G by unpurified cell-free extract of mycelia, there were formed pentoses, phosphotriose, sedoheptulose, and pyruvate [1]. Using R-5-P as substrate we followed the course of the degradation of R-5-P and the formation of PGA during incubation. It was found that the size of the ratio between loss of R-5-P and increase of PGA during the whole period of incubation lay between 2 and 3, which approximately agreed with the stoichiometric ratio in the transketolase reaction of degradation of pentose phosphates (Fig. 2).

For analysis of the products of R-5-P metabolism, the reaction mixture after removal of the proteins was chromatographed on a column of Dowex-1 in the formate form using, for elution, a solvent which contained 2 N HCOOH and 0.05 N sodium formate. The first phosphate ester which appeared in the eluate was identified as S-7-P which contained a small amount of R-5-P. Identification was made by the position of the product on the chromatographic curve, the positive reaction with cysteine and H_2SO_4, and the spectrum of the product of the orcinol reaction. The second peak on the chromatographic curve, judging by the spectrum of the product formed in the reaction with cystine and carbazole according to Dische and Borenfreund [12], was the phosphate ester of a ketopentose, assumed to be Ru-5-P or Xu-5-P (Fig. 3 and 4).

Establishing the presence of the pentose cycle. The above results indicated the presence of transketolase, R-5-P isomerase, and phosphoketopentose epimerase in the cell-free extract. In order to show the presence of the full pentose cycle we took samples at various periods of incubation of the reaction mixture which contained R-5-P as the substrate and followed the change in absorption spectra of the products of the reaction with cysteine and H_2SO_4. After the first five-minute period there was an absorption maximum at 505 mμ and correspondingly at 410 mμ there was indication of formation of sedoheptulose and small quantities of hexose. After 10 min incubation we noted a uniform rise in the amount of both sedoheptulose and hexose. When the period of incubation was lengthened to 30 min, we found a gradual fall in sedoheptulose with simultaneous increase in hexose, which was indicated by the gradual disappearance of the absorption

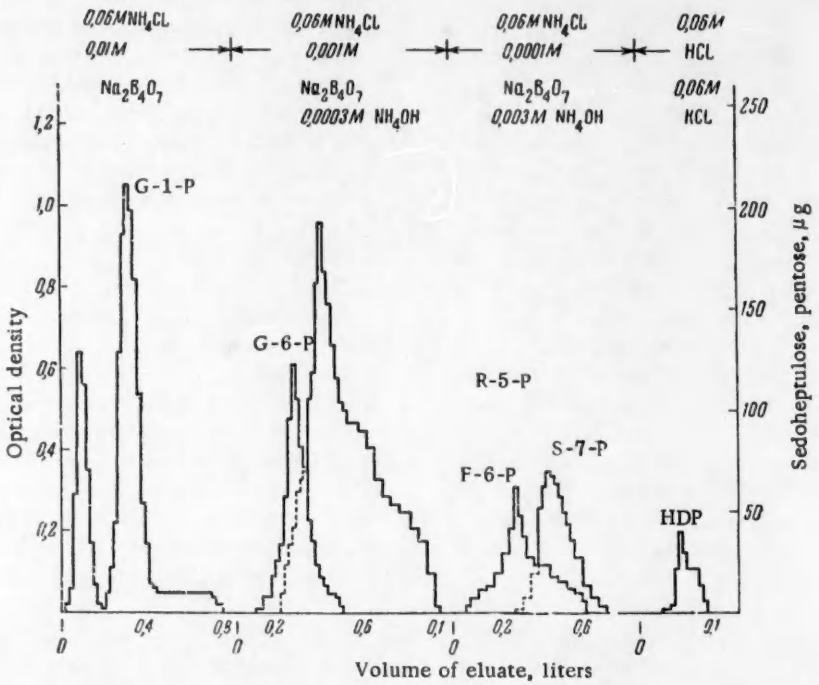


Fig. 1. Separation of sugar phosphate esters by direct ion chromatography. Cell-free extract of mycelia of Streptomyces aureofaciens, chromatographed on Dowex-1 column (Cl^- form).

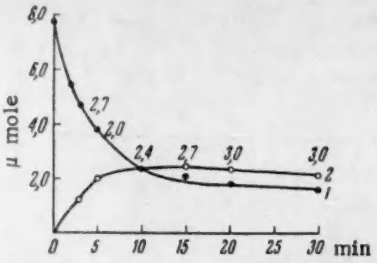


Fig. 2. Transketolase reaction in a cell-free extract of Streptomyces aureofaciens. Composition of sample: 3.1 mg extracted protein, 10 μ mole R-5-P, 20 μ mole $MgCl_2$, 1.5 μ mole cysteine, 0.8 μ mole 0.04 M glycyl glycine solution, pH 7.5 . Total volume 2.5 ml, temperature 37° 1) R-5-P; 2) PGA. Figures on the curve show the ratio R-5-P/PGA

maximum at 505 mμ and the regular increase in the maximum at 410 mμ (Fig. 5). Figure 5 shows that before the beginning of incubation the reaction mixture contained small amounts of hexose introduced with the cell-free extract.

Stoichiometric reaction of transformation of R-5-P. When the cell-free extract was submitted to incubation with R-5-P for five min, we found transformation of 4.1 μ mole R-5-P into 2 μ mole sedoheptulose and 2 μ mole PGA. The molar ratio of disappearing pentose and forming sedoheptulose and PGA agree with the theoretical for a transketolase transformation, namely, from 2 mole R-5-P are formed 1 mole S-7-P and 1 mole PGA. As the incubation was prolonged there was decrease of sedoheptulose corresponding to increase of hexose. The stoichiometric calculation (with a correction for the excess preformed hexose after the first five min of incubation) agreed with the theoretical value for formation of 1 mole of F-6-P from 1 mole of S-7-P, and the total amount of S-7-P and PGA formed during the experiment was equivalent to half the R-5-P used by the system (Table 1).

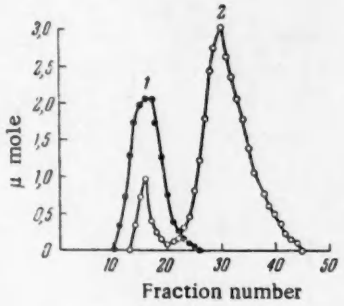


Fig. 3. Separation of metabolic products of R-5-P by ion exchange chromatography. Composition of incubation sample: 15 ml extracted protein, 40 μ mole R-5-P, 25 μ mole $MgCl_2$, 4.6 ml 0.04 M glycyl glycine solution, pH 7.5. Total volume 10 ml, temperature 37°, time of incubation 30 min. The protein-free filtrate of the incubation mixture was chromatographed on a column of Dowex-1 in the formate form (2.2 × 11.5 cm). Volume of each fraction of eluate, 5 ml. 1) Sedoheptulose; 2) pentose.

Oxidative transformation of R-5-P. As noted earlier [1], the activity of G-6-P dehydrogenase is sharply inhibited by orthophosphate but is not inhibited by iodoacetate. On the contrary, for oxidation of PGA the presence of phosphate or arsenate is necessary, and it is suppressed by iodoacetic acid. DPN serves as a hydrogen-acceptor in the enzymatic dehydrogenation of both G-6-P and PGA.

In the experiments with R-5-P as substrate with the presence in the full reaction mixture of DPN, Mg^{++}, cysteine, and other needed components (see note to Fig. 6) we studied the effect of the above-mentioned inhibitors of dehydrogenation on the oxidative transformation of phsophopentoses. With the addition of phosphate or arsenate the rate of oxidation of R-5-P increased in the first ten min, after which, little by little, it fell to a minimum, with accumulation of pyruvate, sedoheptulose, and hexose, Iodacetic acid and NaF scarcely changed the value of the oxidation as compared to the control samples, but inhibited the formation of pyruvic acid (Table 2 and Fig. 6). The results of the experiments showed that R-5-P was oxidized basically by G-6-P dehydrogenase after preliminary transformation into G-6-P; only a small part of the pentose phosphate was oxidized by PGA dehydrogenase at the stage of transformation into PGA by the action of transketolase.

When phosphate or arsenate were added to the system, their inhibiting action on the activity of G-6-P

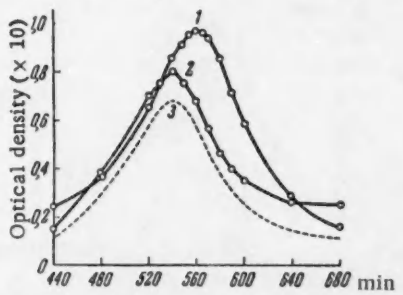


Fig. 4. Absorption spectra of products which form metabolites of R-5-P, determined by the cysteine-carbazole method. 1) Fructose; 2) fraction 30 (see Fig. 3); 3) Xu-1-P (according to Dische).

TABLE 1. Stoichiometry of the Transketolase and Transaldolase Reaction. (Reaction mixture (total volume 2.5 ml) contained: extract (3 mg of protein), 10 μ mole R-5-P, 20 μ mole $MgCl_2$, 1.5 μmole cysteine, 0.8 ml 0.04 M glycyl glycine buffer; pH 7.6, temperature 30°)

Time, min	Micromoles				Ratio	
	R-5-P	PGA	S-7-P	Hexose	$\frac{\text{S-7-P} \times 2}{\text{R-5-P}}$	$\frac{(\text{S-7-P + hexose}) \times 2}{\text{R-5-P}}$
5	—4,1	2,0	2,0	1,0	0,98	1,47
10	—5,5	2,3	2,2	1,3	0,80	1,27
30	—6,4	2,1	1,1	2,2	0,34	1,03
60	—6,5	1,9	0,7	2,7	0,22	1,06

TABLE 2. Analytical Data of Experiments on Oxidation of R-5-P by a Cell-Free Preparation of Streptomyces aureofaciens (composition of sample and experimental conditions shown in Fig. 6

Reaction mixture	O_2 used, μ atom/μ mole	Ribose residue, μ mole	Pyruvate, μ mole	Sedoheptulose, μ mole
Full	1.92	1.3	0.8	0.7
Full without cysteine	0.85	1.2	0	0.1
Full without Mg^{++}	0.78	1.2	0.6	0.5
Full + iodoacetate (5 μ mole)	1.40	1.3	0	0.7
Full + F^- (6 μ mole)	1.50	1.5	0	0.7
Full + AsO_4 (50 μ mole)	0.32	1.7	2.0	1.9
Full + PO_4 (50 μ mole)	0.34	2.9	0.6	1.7

TABLE 3. Analytical Data of the Experiments on Oxidation of F-1,6-P by Cell-Free Preparation of Streptomyces aureofaciens. Composition of samples and experimental conditions shown in Fig. 7. Time of incubation 120 min.

Reaction mixture	O_2 used, μatm/ μ mole	Pyruvic acid, μ mole	Ribose, μ mole	Sedoheptulose, μ mole
Full system	2.54	0.2	0.8	0.1
The same + iodoacetate (5 μ mole)	2.01	0	1.3	0.1
The same + F^- (6 μ mole)	0.15	0	0	0
The same + AsO_4^{---} (50 μ mole)	1.02	2.0	0.1	0
The same + PO_4^{---} (50 μ mole)	0.56	0.1	0.6	0
The same + PO_4^{---} (100 μ mole)	0.42	0.2	0	0

dehydrogenase led to blocking the cyclic transformation of R-5-P, and under these conditions its oxidation took place by PGA dehydrogenase, for the action of which the presence of phosphate was necessary, or, preferably, that of arsenate. The oxidation of PGA as it was formed in the transformation of R-5-P in the presence of phosphate or arsenate led to deficiency in acceptor for the transaldolase reaction; this explains the accumulation of S-7-P. It is still not fully clear why phosphate causes less accumulation of pyruvate than arsenate, and it is not established whether phosphate hastens the further transformation of pyruvic acid.

When iodoacetate or sodium fluoride were added to the system, the inhibition of the action of PGA de-

hydrogenase, or, correspondingly, of enolase, led to blocking the oxidation of PGA. Under these conditions oxidation of R-5-P occurred only under the influence of G-6-P dehydrogenase, and the cyclic metabolism of pentose phosphate was activated. Cysteine is needed for the action of transketolase, and its absence suppressed the formation of sedoheptulose. Magnesium ions have a stimulating effect on the activity of G-6-P dehydrogenase; in their absence the rate of oxidation of R-5-P fell (Table 2).

The results of the studies described above on aerobic and anaerobic degradation of R-5-P demonstrate the functioning of the pentose cycle in mycelia of Streptomyces aureofaciens.

Interrelations and transfer between the EMP and HMP cycles. From the metabolic products of R-5-P we isolated F-1,6-P by ion-exchange chromatography (on a column with Dowex-1 in the formate form):

Eluent	In the eluate, μ moles	
0.2 N HCOOH + 0.03 N HCOONa	S-7-P	13.4
	Pentose-5-P	4.6
0.1 N HCl	F-1,6-P	4.1

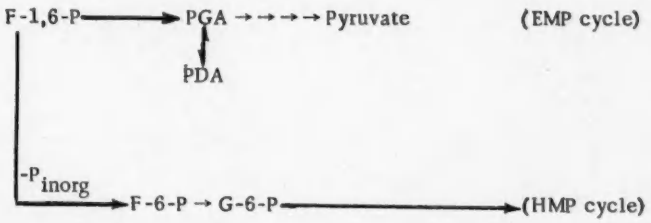
The results of this experiment show that PGA formed in the degradation of R-5-P, beside continuous transformation in the pentose cycle, partly forms F-1,6-P by the action of triose phosphate isomerase and aldolase present in the cell-free extract.

Using F-1,6-P as the substrate for oxidation and analyzing the resulting products of oxidative transformation, we noted the presence of pentose and sedoheptulose along with PGA and pyruvate. This all indicates that F-1,6-P is a general intermediate product in both paths of degradation of the sugars and can undergo oxidation both in the glycolytic (EMP) and pentose (HMP) cycles. The introduction of phosphate into the system does not hasten the oxidation of PGA, but inhibits the action of G-6-P dehydrogenase and this prevents the formation of phosphate esters of pentose and sedoheptulose.

Arsenate, in opposition to phosphate, shows a clearer effect on the rate of oxidation of F-1,6-P. In the first 30 min, arsenate has no inhibiting action on oxidation, but, on the contrary, hastens it, and there is an increased formation of pyruvic acid. Along with this we noted inhibition of the synthesis of phosphoric esters of pentose and sedoheptulose.

These effects, and the action of arsenate on oxygen absorption show that arsenate, on the one hand, stimulates the oxidation of PGA, and on the other, has an inhibiting action on the oxidation of G-6-P. Iodoacetic acid has no effect on the value of the oxidation (use of O_2); it inhibits the formation of pyruvate but does not affect the yield of R-5-P and S-7-P. It is interesting that the addition of NaF to the studied system inhibited the oxidation of F-1,6-P by 90% and completely stopped the formation of R-5-P and pyruvate (Table 3 and Fig. 7).

The above experimental data allow us to conclude that F-1,6-P either is degraded by the EMP scheme, or is first dephosphorylated by phosphatase with succeeding transformation into G-6-P which is further oxidized in the HMP cycle:



F-1,6-P phosphatase with optimum activity at pH 5.0 was noted in the cell-free extract (Fig. 8). The results of the above experiments on oxidation of F-1,6-P, which was inhibited 90% by NaF and was insensitive to iodoacetate, give us reason to assume that in the mycelial cells of actinomycetes, F-1,6-P metabolism proceeds preferably through the pentose cycle.

DISCUSSION OF RESULTS

It was shown that in cell-free extracts of mycelia the pentose phosphate formed in the oxidation of G-6-P undergoes transformation according to the scheme of Horecker. According to Srere [13] and Horecker [14], the acceptor of the glycolaldehyde residue ($H_2COH \cdot HCO$) in the transketolase reaction is Xu-5-P. Therefore the

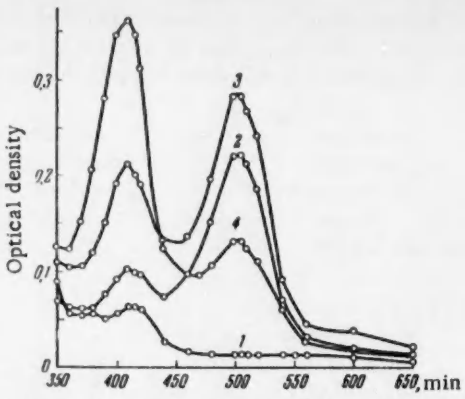


Fig. 5. Curve of absorption of the products which form the metabolites of R-5-P in the reaction with cysteine and H_2SO_4. Composition of reaction mixture: see note to Fig. 2. Incubated at 30°; reaction stopped by addition of 5% TCA; 0.5 ml of filtrate analyzed by the H_2SO_4-cysteine method. 1) before incubation; 2) after 5 min incubation; 3) after 10 min; 4) after 30 min incubation.

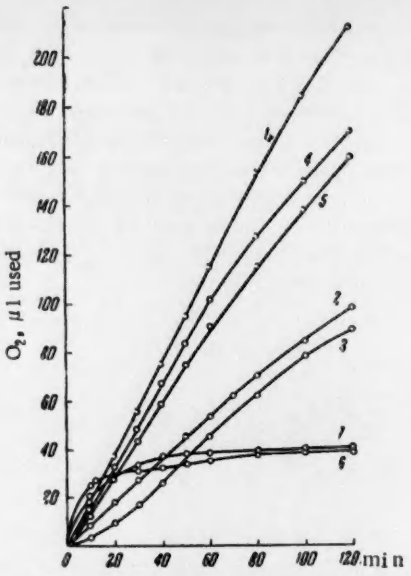


Fig. 6. Oxidation of R-5-P by a cell-free preparation of Streptomyces aurofaciens. Gas metabolism was studied manometrically in a Warburg apparatus at 30° in an air atmosphere. Reaction mixture contained 17.2 mg of extracted protein, 10 µ mole R-5-P, 1 µ mole DPN, 20 µ mole $MgCl_2$, 1.5 µ mole cysteine, 1 µ mole methylene blue 0.8 ml 0.04 M glycyl glycine buffer; pH 7.6. Total volume 2.5 ml. 1) Full system; 2) without cysteine; 3) without $MgCl_2$; 4) added 5 µ mole iodoacetate; 5) added 3 µ mole NaF; 6) added 50 µ mole Na aresenate; 7) added 50 µ mole Na_2HPO_4.

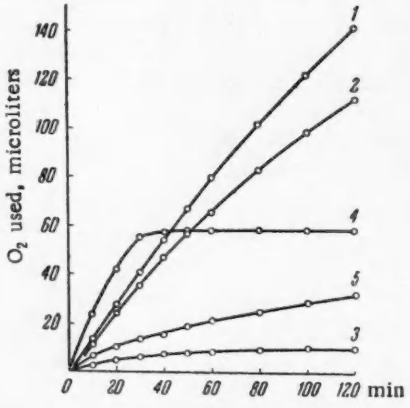


Fig. 7. Oxidation of FDP by cell-free preparations of Streptomyces aureofaciens. Manometric determination of gas metabolism at 30° in air atmosphere. Sample (2.5 ml) contains15.6 mg extracted protein, 5 µ mole F-1,6-P; remaining components as described in Fig. 6. 1) Full system; 2) added 5 µ mole iodoacetate; 3) added 6 µ mole NaF; 4) added 50 µ mole Na arsenate; 5) added 50 µ mole Na_2HPO_4.

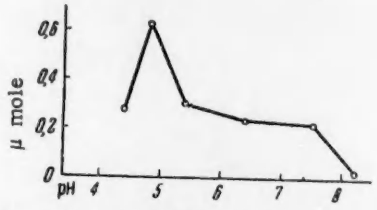


Fig. 8. Activity of F-1,6-P phosphatase of Streptomyces aureofaciens (Reaction F-1,6-P $\rightarrow$F-6-P + P_{inorg}). Reaction system contained in a total volume of 2.5 ml: extract (1.2 mg protein, 5 µ mole F-1, 6-P, 20 µ mole $MgCl_2$, 0.5 ml of one of the buffers mentioned below: 0.1 M sodium acetate - pH 4.4; 4.8; 5.4; 0.4 M glycyl glycine, pH 6.4; 7.5; 0.03 M veronal buffer, pH 8.2. Time of incubation—30 min.

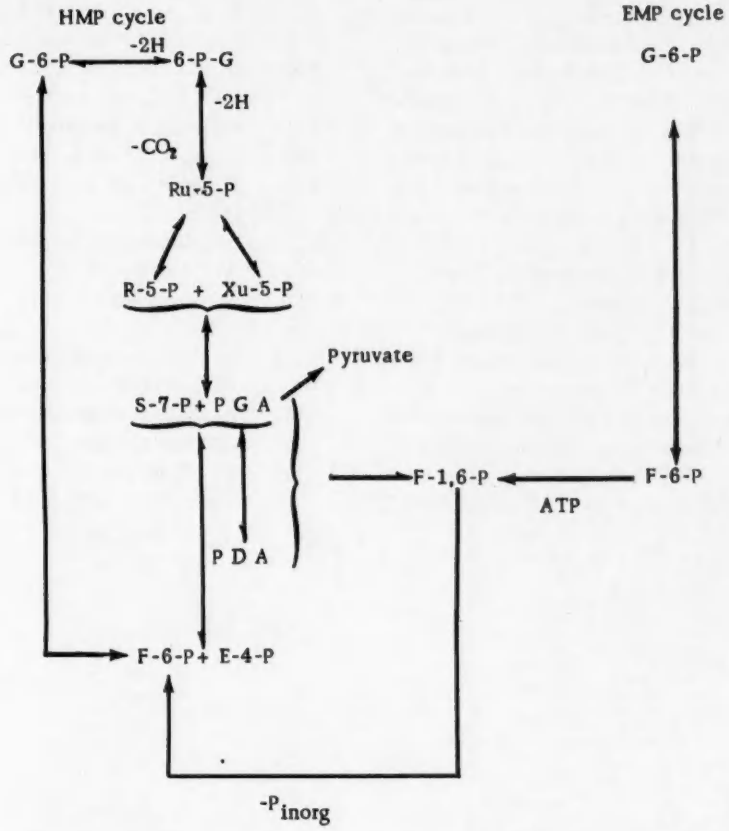
R-5-P used in our experiments as a substrate should, after its transformation by isomerase into Ru-5-P, go over into Xu-5-P under the action of phosphoketopentose epimerase. In the catalyzed transketolase reaction between R-5-P and Xu-5-P, S-7-P and PGA are formed. The formation of S-7-P and PGA under the action of transaldolase results in their again reacting to give F-6-P and E-4-P. F-6-P quickly changes into G-6-P with hexosephosphate isomerase which we [1] observed in cell-free extracts.

Further, the results of studying the transformations of R-5-P both anaerobically and aerobically (in the presence of methylene blue) give a basis for the conclusion of the actively functioning pentose cycle in the mycelia cells of Streptomyces aureofaciens.

When pentosephosphate is transformed, hexosemonophosphate and F-1,6-P appear. The experiments showed the presence of competition between aldolase and transketolase on the intermediate product, PGA.

Both alternative paths for the transformations of sugars are connected through the common intermediate metabolite, fructose diphosphate (scheme):

Scheme of Interrelations of Glycolytic (EMP) and Pentose (HMP) Cycles in Streptomyces aureofaciens



The question of which of the two cycles, EMP or HMP, is the preferred path for transformation of the sugars in Streptomyces aureofaciens requires further study. The connecting metabolite between both alternative systems is F-1,6-P; it can either be degraded after dephosphorylation, transformed by the scheme of the pentose cycle, or undergo oxidation through the EMP system. Here orthophosphate plays an important role. In view of the demonstrated inhibiting action of phosphate on G-6-P dehydrogenase, its presence can shift the transformation of F-1,6-P from the pentose to the glycolytic path. We can conclude from this that the concentration of phos-

phate ion in the culture medium can affect the functioning of one or the other cycle of degradation of sugars in Streptomyces aureofaciens.

The relations and interrelations between the two alternative paths of carbohydrate metabolism are very important for biochemists. The basic factor which determines the functioning of the hexose monophosphate cycle or the Embden-Meyerhof-Parnas cycle is usually considered to be the presence or absence of phosphohexokinase. This investigation has shown that along with this, orthophosphate is of great significance for the direction of transformation of sugars. These results can be of interest in relation to the regulation of metabolism not only in microorganisms, but also in other, higher organisms.

SUMMARY

Cell-free extracts of Streptomyces aureofaciens under anaerobic conditions transform ribose-5-phosphate (R-5-P) with formation of sedoheptulose-7-phosphate (S-7-P), triosephosphate (PGA), and hexose phosphate.

The functioning of the pentose cycle is confirmed by the fact that with short incubation we find transformation of R-5-P into S-7-P and PGA in a ratio which corresponds to the theory for the transketolase reaction (2:1:1). In the corresponding mechanism of oxidative transformation of R-5-P (in the presence of methylene blue), there is inhibition by phosphate or arsenate and relative insensitivity to iodoacetate.

We have established the formation of fructose-1,6-diphosphate (F-1,6-P) in the transformations of R-5-P. F-1,6-P can be transformed in the system studied by the glycolytic pathway, or can be dephosphorylated by the phosphatase of the extract into F-6-P which after conversion to G-6-P is oxidized through the pentose cycle. Both alternative paths of degradation of carbohydrates are connected through F-1,6-P, as the common intermediate product.

We have shown that phosphate has an inhibiting action on the G-6-P dehydrogenase of Streptomyces aureofaciens [1]; on the other hand, phosphate is required for the action of triosephosphate dehydrogenase. The results obtained in this study of oxidative degradation of F-1,6-P in cell-free extracts of the mycelia indicate that the presence of phosphate can shift the degradation of F-1,6-P from the glycolytic to the pentose path.

LITERATURE CITED

[1] Shen Sang-ch'ung et al., Acta Physiol. Sci 21, 302 (1957).

[2] B.L. Horecker, P.Z. Smyrniotis and H. Klenow, J. Biol. Chem. 205, 661 (1953).

[3] Z. Dische, J. Biol. Chem. 204, 983 (1953).

[4] Z. Dische, L.B. Shettles and M. Osnos, Arch. Biochem. 22, 169 (1949).

[5] J.H. Roe, J. Biol. Chem. 107, 15–22 (1934).

[6] O. Meyerhof and K. Lohmann, Biochem. Z. 271, 89 (1934).

[7] E.C. Slater, Biochem. J. 53, 157 (1953).

[8] S. Racker, J. Biol. Chem. 167, 843 (1947).

[9] K. Burton and T.E. Wilson, Biochem. J. 54, 86 (1953).

[10] J.S. Khym and W.E. Cohn, J. Am. Chem. Soc. 75, 1153 (1953).

[11] B.L. Horecker et al., J. Biol. Chem. 193, 383 (1951).

[12] A. Dische and E. Borenfreund, J. Biol. Chem 192, 583 (1951).

[13] P.A. Srere et al., Arch. Biochem. and Biophys. 59, 535 (1954).

[14] B.L. Horecker, P.Z. Smyrniotis and J. Hurwitz, J. Biol. Chem. 223, 1009 (1956).

THE AMPHOTERIC PROPERTIES OF THE ACTIN-LIKE PROTEIN FROM THE SMOOTH MUSCLE OF DOG STOMACH

V.V. Oppel' and T.B. Khlyustina

Institute of Evolutionary Physiology, Academy of Sciences USSR, Leningrad

Translated from Biokhimiya Vol. 25, No. 3, pp. 532-539, May-June, 1960

Original article submitted Sept. 10, 1959

During our work on the structure of the smooth muscle (stomach) proteins of dogs we observed [1], in the salt extract (0.5 M KCl, pH 7.0-7.1) from this muscle, a protein which precipitated in the zone of $(NH_4)_2SO_4$ concentration characteristic for the salting-out of actin in striated muscle (10-20% saturation of $(NH_4)_2SO_4$).

The presence of a salting-out peak characteristic of actin has been described by other authors for a contractile complex of proteins precipitated from a salt extract by its 10-20-fold dilution with water for proteins extracted from the smooth muscle of the uterus [2] and the smooth muscle of pig esophagus. The Swedish and Japanese authors considered this protein to be actin, although they observed that the actin of smooth muscle differed somewhat in its properties from the actin of striated muscle.

In work from our laboratory [4] it was shown that the actin-like protein of dog stomach smooth muscle had a low specific viscosity and a small value of anomalous viscosity which lay in the limits characteristic of depolymerized G-actin. The viscosity of the solution of this protein did not change under the influence of added ATP, a fact which indicates that it is not a contractile protein complex. The actin-like protein of smooth muscle, dialyzed free of $(NH_4)_2SO_4$ with careful protection of its active group [5], gave a sharp increase in viscosity (formation of actomyosin complex!) when its solution was mixed with a solution of myosin extracted from striated rabbit muscle [4]. Thse experiments showed that the actin-like protein isolated from smooth muscle belonged to the family of "actins". It is important that it be further identified with the actin of striated muscle.

For this purpose we decided to determine the number of ionogenic groups in it and also its isoionic point. The amino acid composition of actin differs quite sharply in number of dicarboxylic and diamino acids from the composition of myosin and tropomyosin [6-9]. This permits us to expect evidence from the results of electrometric titration of the ionogenic groups of the actin-like protein of smooth muscle. On the other hand, establishing the isoionic point of the protein will permit calculations to reveal the same difference which was found between the isoelectric points of actin, myosin, and actomyosin by the method of electrophoresis [10].

MATERIALS AND METHODS OF STUDY

Protein preparations. The actin-like protein of smooth muscle was extracted along with other proteins in cold from a homogenate of dog stomach muscle by 0.5 M KCl mixed with phosphate buffer (0.01 M KH_2PO_4 and 0.02 M Na_2HPO_4, pH 7.0-7.2). The ratio of volume of extracting liquid to weight of muscle homogenate was 5:1. After standing in the cold for a day, the liquid was filtered through linen and dialyzed for a day in a collodion bag (in the refrigerator) against a solution of 0.5 M KCl with a phosphate buffer, pH 7.0-7.2.

The dialyzed extract was salted out at room temperature with a saturated solution of $(NH_4)_2SO_4$ and neutralized by addition of ammonia to pH of 7.0-7.1. Aggregation of the salted-out protein lasted 15-20 min, after which the precipitate was separated by centrifuging, and dissolved in 0.5 M KCl. The resulting solution was dialyzed overnight in a refrigerator against 0.5 M KCl. For further purification [4] we subjected the dialyzed protein solution to repeated salting out. In the first salting-out the concentration of the $(NH_4)_2SO_4$ solution was brought to 20% of saturation; in the second, attempting to obtain actin less contaminated by admixtures of other proteins, we used a less concentrated solution (15% of saturation). After 15-20 min the precipitate was again separated by centrifuging, dissolved in 0.5 M KCl, and dialyzed in the cold against 0.5 M KCl to complete removal of NH_4^+ (Nessler reaction). In order to hasten the dialysis, the outer solution was changed every 2-3 hours.

The protein content of the solution was determined by the Conway method as modified in our laboratory ($N \times 6.11$). Its content varied between 0.30 and 0.50%. These protein solutions were submitted to further study.

Actomyosin of dog striated muscle was precipitated from a salt extract prepared in the same way (0.5 M KCl + phosphate buffer, pH 7.0-7.2) by tenfold dilution with water. The precipitate, separated by centrifuging, was dissolved in 0.5 M KCl and three times purified by precipitation by dilution with water and re-solution in

0.5 M KCl. The thus-purified protein solution was dialyzed for a day against 0.5 M KCl. The protein content in the solution was determined by nitrogen (N × 6.11). The actomyosin solutions contained 0.35-0.50% protein.

Determination of the isoionic point of the proteins. Since the exact determination of the isoionic point of the studied proteins was necessary for us to calculate the number of ionogenic groups in the studied proteins, we could not be limited to graphic interpolation from the titration curve. For determination of the isoionic point we used the method of determination of the pH of the solution (suspension) of protein [11] submitted to full dialysis against distilled water. The determination of pH in these experiments, like determinations of it in all experiments with electrometric titration, was carried out with a potentiometer of type LP-5 with a glass electrode.

Electrometric titration was carried out as follows: 10 ml of solution of the protein dissolved in 0.5 M KCl was mixed with 10 ml of 0.1 N HCl. The resulting acid solution was titrated with a solution of 0.5 N KOH which was added from a microburet in small amounts, 0.2 ml at the beginning and end of the titration, 0.02 ml in the zone of slight buffering of the protein solution. The alkali was prepared in water freed from CO_2 and kept in a vessel sealed from penetration by CO_2. After each addition of alkali we determined the pH of the solution. In the acid zone the potential was established quickly. In the alkaline region it was sometimes necessary to wait 10-15 min to establish a constant potential. Since the vessel in which titration took place was not protected from penetration of CO_2 the results of the titration in the alkaline region were less exact. In view of the small volume of alkali added, correction for it was not made. We always carried out parallel titrations of 10 ml of 0.1 N HCl mixed with 10 ml of 0.5 M KCl. This permitted us to make graphic calculations without making corrections for activity of the hydrogen ion. It was assumed that the activity of H^+ in solutions with and without protein was the same.

At the beginning of the work we tested the method of determining the pH in separate samples of protein solution mixed with 0.1 N HCl and neutralized by addition of different amounts of alkali in each sample. The volume of each sample was equalized at exactly 20 ml. However, we were unable to use this more exact method because of lack of protein material isolated from dog stomach. Either it was necessary in each experiment to determine only part of the curve, or to take a small amount of protein in each analysis.

Before titration of the protein solutions we ran a preliminary titration of solutions of 0.01 N HCl (12 experiments), 0.1 N H_3PO_4 (4 experiments), and 0.5% glycine (15 experiments).

We also carried out experiments on electrometric titration of the amino groups of proteins and glycine. In our modified method, after a number of experiments, formalin, neutralized to pH 7.0-7.1, was added to a solution of protein titrated to pH 9.6 in an amount which gave a 2 M concentration of it in the solution. Then titration was repeated and it was again brought to pH 9.6. In the same way we carried out parallel titration of a mixture of 0.1 N HCl + 0.5 M KCl, to which neutralized formalin was added.

Calculations. The resulting pair of titration curves, the curve of pure acid and of acid + protein, was plotted on the same sheet of millimeter paper (the number of milliliters of alkali used on the abscissa, the observed pH value on the ordinate). Then the points were found, on the curve, which had the same pH. The difference between the corresponding points on both curves expressed in milliliters of KOH (0.5 N) allowed us to calculate the amount of bound (or given off) equivalents of H^+ for the protein. We made this graphic calculation for all the experimental points on the curve which permitted use to construct a curve of binding H^+ by protein at different pH values. Knowing the concentration of the protein in 1 ml of solution, we expressed the results of the experiments, as is usually done, in the form of binding H^+ by the protein in the amount of 10^5 g. Analogous calculations were carried out also for titration of the protein in the presence of formol.

The determination of the number of ionogenic groups in the protein was also carried out graphically, as indicated in the literature [12-19]: The number of equivalents of total bases was determined from the isoionic point to the point of maximum binding of acid; the number of equivalents of all the free carboxyls is from the point of maximum binding of acid to pH 5.6--6.0, the number of equivalents of Σ-NH_2 groups of lysine by difference between titration in the absence and presence of formol at pH 9.6.

Since we did not carry out the titration at different temperatures or in the presence and absence of salt in the solution, the electrometric titration could not give us the content of equivalents of the imidazole groups of histidine. Hence, we could not calculate the guanidine groups of arginine.

RESULTS

Measurement of the isoionic point of the stomach smooth muscle actin-like protein and the actomyosin of striated muscle carried out by the method of complete dialysis gave the following results:

Actin-like protein, pH	Actomyosin, pH
5.25	5.60
4.95	5.30
4.75	5.70
5.10	–

The resulting data show that the isoionic point of the actin-like protein is shifted to the acid side of the isoionic point of actomyosin by 0.5 pH units. This difference was found in all the experiments. The isoionic

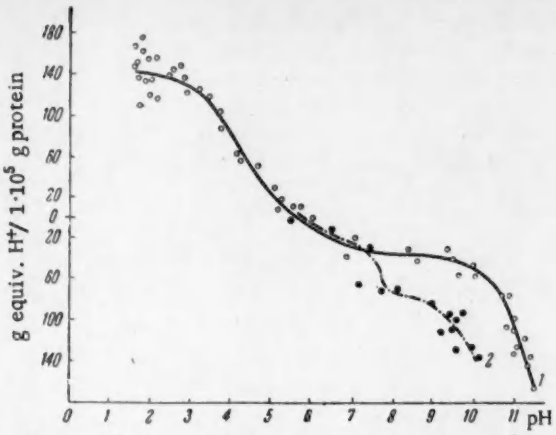


Fig. 1. Binding of H^+ by actomyosin (g-equiv./10^5 g protein) 1) Titration by alkali; 2) titration by alkali in the presence of formol.

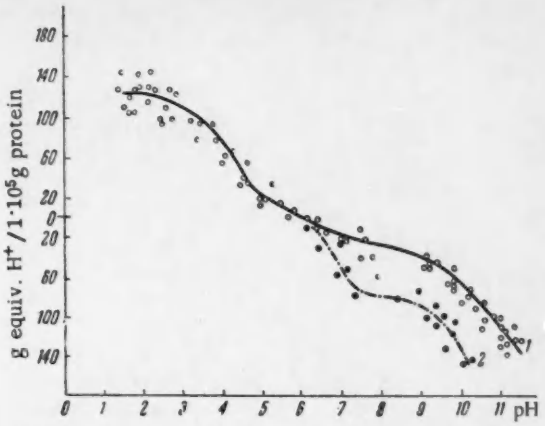


Fig. 2. Binding of H^+ by the actin-like protein (g-equiv./10^5 g protein). 1) Titration by alkali; 2) titration by alkali in the presence of formol.

point for actomyosin which we found corresponds to the literature data [20-22]. The isoionic point for the protein of smooth muscle agrees with the isoelectric point of G-actin [23].

Determination of the maximum number of equivalents of H^+ bound by protein in the electrometric titration presented some technical difficulties because of the great jump in potential found in the pH region more acid than pH 1.9-2.0. In this region the buffer capacity of the protein had already practically been exhausted. In connection with this we devoted particular attention to the determination of this section of the titration curve, which had great value for later calculations. Graphical extrapolation of the results on the ordinate gave the desired value in g-equiv. H^+ bound by 10^5 g of protein.

In the experiments on titration of the actomyosin of striated muscle we showed that 10^5 g of this protein, obtained from dog muscle, bound 146 g-equiv. H^+ (Fig. 1). This is sufficiently close to the value of 156 g-equiv. H^+ given by Dubuisson [19, 24] for the actomyosin of rabbits and cats, and differs considerable from the figure given by Goryukhina [25] for the actomyosin of fish (243 g-equiv. per 10^5 g protein).

Calculation of the ionogenic groups shows that the total bases in 10^5 g of dog actomyosin have 141 g-equiv., free carboxyls have 154 g-equiv., and Σ-NH_2 of lysine have 75 g-equiv.

Titration of the actin-like protein of smooth muscle shows (Fig. 2) that the maximum binding of H^+ is 124-125 g-equiv. per 10^5 g of protein. The number of ionogenic groups calculated graphically from these values and from the position of the isoionic point of the protein (pH 5.01) is for total bases 108 g-equiv., for free carboxyls, 124 g-equiv., and for Σ-NH_2 of lysine, 49-50 g-equiv. per 10^5 g of protein.

We did not calculate the maximum binding by the protein of the OH^- ions as the previous authors did [24, 25]. Titration in the alkaline region did not give the final binding of OH^-, so extrapolation becomes very arbitrary. Also, titration in the strongly alkaline region gives irreversible results, which indicates the occurrence of denaturation of the protein.

DISCUSSION OF RESULTS

Comparison of the number of ionogenic groups in actomyosin obtained by us by electrometric titration with the literature data obtained by the same method is made difficult by the fact that the authors do not give such calculations. Only from the work of Dubuisson [20] can be obtaine the g-equiv. values for Σ - NH_2 of lysine obtained by formol titration. It is 73 and agrees well with our value of 75 g-equiv. per 10^5 g of actomyosin. The data of Goryukhina [25] for formol titration cannot be used because of incomplete experimental data needed for calculation.

The results obtained by electrometric titration are compared (Table 1) with the data of amino acid analysis of actomyosin. Unfortunately, the data on the composition of actomyosin in the literature do not have sufficient accuracy. The older work [26, 27] underestimated the amino acids in the protein by about 15% and so is little suitable for comparison. In recent times, analyses of actomyosin have not been made because of the complex and, as it is assumed, inconstant composition of this protein.

For comparison withour data we also used the more up to date and accurate analysis of the amino acid composition of myosin and of actin of rabbits [28], considering that the actomyosin of striated muscle of warm-blooded animals contains myosin and actin in the ratio 4:1 [29].

For calculation of the ionogenic groups, the sum of all the bases was calculated by addition of the equivalents of arginine, lysine, and histidine.

TABLE 1. Content of Ionogenic Groups (g-equiv.) in 10^5 g of Actomyosin

Ionogenic groups	Electrometric titration		Amino acid analysis	
	our data	data of dubuisson	Actomyosin †	myosin/actin 4:1‡
Free carboxyl	154	156 •	123	147
Total bases	141	148 •	114	132
Σ-NH_2 lysine	75	73	68	79

• Calculated by us

† According to the literature [26, 27]

‡ According to the literature [28, 29]

Free carboxyl groups were calculated as the sum of the equivalents of aspartic and glutamic acids after subtracting the equivalents of amide nitrogen.

As Table 1 shows, the results of electrometric determination of the ionogenic groups of actomyosin obtained by different investigators agree excellently.

The fact that we isolated our actomyosin from the striated muscles of dogs, and Dubuisson and Hamoir [24] obtained theirs from muscles of rabbits and cats indicates the great similarity in structure of this protein in different orders of mammals and also the constancy of composition of actomyosin as an individual, though complex, protein.

The data on the content of ionogenic groups obtained by electrometric titration and that calculated from the amino acid composition of the mixture of 4 parts of myosin and 1 part of actin differs somewhat. All the same, a difference of 7 g-equiv. of carboxyls, 9 g-equiv., of bases, and 4 g-equiv., of Σ-NH_2 for lysine cannot be considered appreciable. A different picture is obtained on comparing the results of electrometric titration with the data of the older analysis of rabbit actomyosin. However, these analyses, as already mentioned, cover only 85% of the amino acids in the protein.

Turning to the analysis of the actin-like protein of smooth muscle, we confirm that the difference from actomyosin observed in its investigation is quite real. Naturally, the comparison of the number of ionogenic groups which it contains must be made with their content in the actin of striated muscle, with which protein it has a number of common properties [1, 4]. The absence of work on the electrometric titration of actin makes it necessary to compare only the results of determination of the amino acid composition of actin [7, 9].

The comparison of these values is given in Table 2. We note the great similarity in our results and the results of the analysis of the American group of investigators. The analyses of Ivanov and Asmalova [7] are higher with respect to lysine, which is indicated not only by the better agreement of the data for this amino acid from us and from the American investigators, which gives a figure almost 10 g-equiv. lower than in the work of Ivanov and Asmalova, but in still greater degree by the somewhat higher sum of the bases in the work of the latter workers over the sum of the free carboxyl groups; this is entirely incorrect (correction for the amide groups of the dicarboxylic amino acids was correspondingly introduced in the work of Kominz et al. [9]).

Consideration of the data of Table 2 leads to the conclusion that in number of ionogenic groups and also from the region of salting out by $(NH_4)_2SO_4$, from viscosity, and from ability to form the actomyosin complex, the

TABLE 2. Content of Ionogenic Groups (g-equiv.) in 10^5 g of Actin-Like Protein of Smooth Muscle and Actin of Striated Muscle

Ionogenic groups	Electrometric titration	Amino acid analysis	
	our determination	K ommz et al.	Ivanov and Asmolova
Free carboxyl	124	117	115
Total bases	108	109	123
Σ- NH_2 lysine	50	52	61

actin-like protein of smooth muscle does not differ appreciably from the actin of striated muscle. The similarity is completed by the position of the isoionic point (pH 5.01) which agrees with the isoelectric point of depolymerized actin [23].

Hence we conclude that the protein of smooth muscle isolated from it by salt extraction and salting-out with $(NH_4)_2SO_4$ at a concentration of 10-20% of saturation is actin. Solution of the question of whether the actin of smooth muscle is identical with the actin of striated muscle or differs from it by some amino acid residue, as is found in the tropomyosin isolated from different muscles [9], would require special investigation.

In the work of Goryukhina [25] along with data on actomyosin, myogen and globulin X obtained from the muscular tissue of fish, there are given data on electrometric titration of the myostromin of Danilevskii. Although the calculated number of ionogenic groups in this protein, as in the case of fish actomyosin, is impossible, due to insufficiently careful establishment of the maximum binding of H^+ by this protein, yet it is obvious there is less binding of H^+ by it than by actomyosin: the difference at pH 1.85-1.96 is 54 g-equiv. per 10^5 g of protein. In our experiments with the muscle protein of dogs we found a difference in maximal binding of H^+ in the actomyosin of striated muscle and the actin isolated from stomach muscle of 22 g-equiv.

The question arises whether myostromin is not actually actin. This opinion has already been expressed by Raiko [30]. In Table 3 we compare the various properties of myostromin and actin.

Consideration of Table 3 shows the unusual similarity of both proteins. Their participation in the structure of muscle fibers, their content in muscle, and their isoelectric points, found in a considerably more acid position than the isoelectric point of actomyosin, the great similarity of content in the muscle of both proteins, closeness of sum of total bases, markedly lower ability than actomyosin to bind H ions, all these show the great resemblance of these proteins. The difficulty of purifying both myostromin and actin from nonprotein contaminants, shown by the low nitrogen content, indicates their strong bonds with other organic compounds (carbohydrates [30]) in muscle tissues. Finally, the appearance in the prosthetic group of myostromin of a purine nucleotide with more than 1.5 moles of phosphoric acid per 1.0 mole of adenine [30] evidently corresponds to the adenine diphosphate found as a prosthetic group in actin [5, 34]. The very low viscosity of myostromin solutions indicates its full denaturation which occurs, as in actin [5], with great ease.

Summarizing the properties of actin and myostromin considered in parallel, we come to the conclusion that Danilevskii and his school long ago had in their hands

TABLE 3. Comparison of the properties of Myostromin and Actin

Properties	Myostromin	Literature data	Actin	Literature data
Microscopic structure of muscle fibers (myosin first removed)	Before extraction of the protein, retained. After extraction, absent	[31]	Before extraction of the protein, retained After extraction, absent	[32]
Content in muscle (% of total protein).	7 – 14.4	[30]	13 – 15	[33]
Content of N, %	14.7	[30]	15.2	[34]
			16.2	[5]
Content of phosphorus in prosthetic group, mole ratio phosphorus/adenine	>1.5	[30]	2	[34]
Relative viscosity	1.05 – 1.09 (1%)	[35]	1.20 (0.5%)	[5]
Isoelectric point, pH	5.2	[25]	5.01 – 5.10	[23]
	5.2 – 5.4	[35]	(G-actin)	
Sum of total bases, g-equiv. 10^5 g protein	99	[36]	108	Our work
			109	[9]
Difference between maximum binding of H+ in actomyosin and actin, g-equiv. 10^5 g protein	54 (not exact)	[25]	22	Our work

this protein (actin) whose share in the reaction with myosin could form the contractile protein complex protein actomyosin. Even the participation of myostromin (actin) in muscular contraction was foreseen by Danilevskii, although evidence for this was impossible due to absence of suitable methods. Certainly myostromin is a strongly-denatured protein preparation. We can regret that the study of myostromin, so successfully begun, was stopped because of the publication of the work of Szent-Gyorgyi and his school.

In conclusion we wish to note the great usefulness of the method of electrometric titration for the determination of ionogenic groups in proteins. It gives good characterization of individual proteins. Among the structural proteins of muscle it permits the separation from each other of actomyosin([19, 20, 24] and the present work), myosin [16], and separately of both of its components, H- or L-meromyosin [17], and also of actin (present work). It offers the possiblity of identifying protein with one of the components of a protein mixture from which this protein was isolated. This was done in the present work.

SUMMARY

The protein salted out of a salt extract of smooth muscle by $(NH_4)_2SO_4$ in a concentration of 10-20% of saturation is shown, by electrometric determination of its ionogenic groups, to be idential with actin.

Actomyosin of striated muscle of dogs shows the same number of ionogenic groups as actomyosin from the muscles of rabbits and cats.

The myostromin of A.Ya. Danilevskii is evidently denatured actin.

LITERATURE CITED

[1] V.V. Oppel' and T.P. Serebrenikova, Doklady Akad. Nauk SSSR 122, 271 (1958). *

[2] O. Snellman and M. Tenow, Biochim et biophys. acta 13, 199 (1954).

[3] I. Tonomura and A.T. Sasaki, Enzymologia 18, 111 (1957).

[4] V.V. Oppel' and T.P. Serebrenikova, Biokhimiya 24, 648 (1959).*

[5] T. Tsao and K. Bailey, Biochim. et biophys. acta 11, 102 (1953).

[6] G. Feuer, T. Molnar, E. Pettko, and F. Straub, Hung. Acta Physiol. 1, 150 (1948).

[7] I.I. Ivanov and E.I. Asmolova, Biokhimiya 15, 201 (1950).

[8] A.V. Gerasimova and I.I. Ivanov, Voprosy Med. Khim. 5, 110 (1953).

[9] D.R. Kominz, A. Hough, P. Symonds, and K. Laki, Arch. Biochem. and Biophys. 50, 148 (1954).

[10] G. Hamoir, Disc. Faraday Soc. 13, 116 (1953).

[11] F. Olberti in: Proteins 2, edited by G. Neurath and K. Bailey [Russian translation] (IL, Moscow, 1956), p. 446.

[12] R.K. Cannan, A. Kibrick, and A.H. Palmer, Ann. N. Y. Acad. Sci. 41, 243 (1941).

[13] R.A. Kekwick and R.K. Cannan, Biochem. J. 30, 227 (1936).

[14] R.K. Cannan, A.H. Palmer, and A.C. Kibrick, J. Biol. Chem. 142, 803 (1942).

[15] C . Tanford, J. Am. Chem. Soc. 72, 440 (1950).

[16] E. Mihalyi, Enzymologia 14, 224 (1950).

[17] L.R. Naninga, Arch. Biochem. and Biophys. 56, 349 (1955).

[18] R.A. Kekwick and R.K. Cannan, Biochem. J. 30, 235 (1936).

[19] M. Dubuisson, Arch. intern. physiol. 51, 154 (1941).

[20] M. Dubuisson, Arch. intern. physiol. 51, 133 (1941).

[21] W. Holwede and H. Weber, Biochem. Z. 295, 205 (1938).

[22] H. Weber and H. Portzehl, Adv. in Protein Chem. 7, 161 (1952).

[23] V. Debain, Biochem et biophys. acta 21, 182 (1957).

[24] M. Dubuisson and G. Hamoir, Arch. intern. physiol. 53, 308 (1943).

[25] T.A. Goryukhina, Dissertation: Characteristic Amphoteric Properties of Muscle Proteins of Fish, (Kiev, 1945).

[26] K. Bailey, Biochem. J. 31, 1406 (1937).

[27] I.G. Sharp, Biochem. J. 33, 679 (1939).

[28] K. Bailey, Biochem. J. 43, 271 (1948).

[29] I. Gergely and M.A. Gouvea, Federat Proc. 12, 207 (1953).

[30] Z.A. Raiko, Dissertation: The Form of Iron Compounds in the Muscle Protein Myostromin, (Samarkand, 1943).

[31] K. Shepilova and A.Ya. Danilevskii, Z. Physiol. Chem. 5, 349 (1881).

[32] H.E. Huxley, Biochim. et biophys. acta 12, 387 (1953).

[33] W. Hasselbach and G. Schneider, Biochem. Z. 321, 462 (1951).

[34] W.F.H.M. Mommaerts, J. Biol. Chem. 198, 445 (1952).

[35] G.E. Vladimirov, Biochem. Z. 222, 135 (1930).

[36] A.L. Yaroslavtsev. Trudy Voennomeditsinsk. Akademii 22, 17 (1940).

*Original Russian pagination. See C.B. Translation.

THE EFFECT OF IONIZING RADIATION ON THE PHOSPHOLIPID METABOLISM IN THE LIVER

A.S. Kainova

Translated from Biokhimiya Vol. 25, No. 3, pp. 540-544,
May-June, 1960
Original article submitted September 12, 1959

The phospholipid metabolism is of great importance to the life phenomena in all tissues of the animal organism. Phospholipids form a part of the lipoprotein complexes in mitochondria and microsomes, the biological structures which are the centers where the enzymatic activity of the cells is concentrated, they participate in the formation of cell membranes, affecting their permeability and other forms of biological activity. Phospholipids are present in the prosthetic group of many enzymes (the succinoxidase system, the adenosine triphosphatase, which is activated by magnesium, the choline oxidase, etc.). Changes in phospholipids under the influence of ionizing radiation are therefore of great interest.

It is stated in the scientific literature that the total content of phospholipids in rat liver does not change following irradiation [1-3]. The total amount of phospholipids remains constant also in the mitochondria and microsomes of rabbits [4]. However, the total content of phospholipids in dog's liver increases following irradiation [5], and decreases in the brain of rats [6]. According to some authors [7], the intensity of phospholipid exchange in rat's liver is not affected, but according to others [8, 9] it is increased, while in rat's brain it is decreased [8].

The present paper deals with an investigation of early changes in various fractions of phospholipids in the liver of rats during the first day after a general single irradiation with gamma rays.

METHODS

In the first series of experiments 49 male rats of 180-220 g body weight were used. Out of these, 22 served as controls, and 27 were subjected to a single general irradiation with gamma rays, the dosage amounting to 1000 r from an EGO-2 apparatus, and a dose potency of 460 r/min. The animals were killed 2, 4, 6 and 24 hours after irradiation, in groups of six for each period.

In the second series of experiments we studied the powerful effect of gamma rays on the phospholipid exchange of the liver in rabbits, which was observed immediately after the end of irradiation. The experimental animals were killed 10, 30 and 60 minutes after the start of irradiation, the corresponding dosage amounting here to 5000, 15,000 and 30,000 r. Each group consisted of five animals, and five rabbits served as controls.

$Na_2HP^{32}O_4$, in an amount of 0.1 mCurie/200 g body weight, was introduced intraperitoneally one hour previous to the killing. After decapitation, the liver of the animals was rapidly excised, and the phospholipids extracted by grinding the liver 6 - 7 times with chloroform and ethyl ether (2:1) in a mortar. After each grinding the tissue homogenate was centrifuged and filtered. The obtained extracts were poured together, and the inorganic phosphorus removed by means of 0.1 and 0.01 N HCl.

The resulting phospholipid extract was evaporated in a vacuum in an atmosphere of nitrogen, at 40-50°C. The dry phospholipid residue was dissolved in methyl alcohol, thus removing the inositol phosphatides which are insoluble in this medium. A further separation of the phospholipid into fractions was conducted by the method of Dawson [10] and Smirnov [11]. The cephalin fraction was adsorbed from the methanol solution by MgO, the lecithin and sphingomyelin fractions remaining in solution. The separation of the lecithins and sphingomyelins was conducted by means of alkaline hydrolysis of the lecithins with 1 N NaOH, with incubation at 37°C for 20 hours. In order to obtain the cephalin fraction, the MgO was dissolved in 1.5 N HCl, after which the cephalins were extracted by chloroform. As a result, the following fractions were obtained: the glycerophospholipids lecithins and cephalins, consisting of colamine, serine, and acetylphosphatides, sphingomyelins and inositol phospholipids.

Samples were taken from each fraction for the determination of inorganic and radioactive phosphorus (the radioactivity being measured by Geiger-Muller's end counter). The ashing of the samples was accomplished by means of concentrated sulfuric and nitric acids. Phosphorus was determined by Fiske and Subbarow's method. The phospholipids were calculated by multiplying the obtained P value (in mg) by the factor 23.5.

RESULTS AND DISCUSSION

The data represented in Table 1 show that the total amount of phospholipids in the liver of rats does not change, as compared with the normal value, during 2,4,6 and 24 hours following irradiation. As to the content of various phospholipid fractions, glycerophospho-

TABLE 1. Total Phospholipids and Their Fractions in Rat Liver Prior and After Total Gamma-irradiation

Time after irradiation, hr.	Total phospholipids in % of raw weight	Phospholipid fractions (in % of total)			
		lecithins	cephalins	inositol phosphatides	sphingomyelins
Control	2,6±0,1	55,4±1,8	30,1±1,2	1,4±0,1	2,4±0,2
2	2,4±0,1	58,5±2,8	30,4±1,5	1,2±0,2	2,1±0,6
4	2,7±0,1	53,9±3,9	34,3±4,0	1,4±0,1	2,1±0,3
6	2,4±0,1	53,0±4,5	33,1±2,6	1,3±0,2	2,8±0,4
24	2,6±0,1	74,1±1,4	13,0±1,6	1,1±0,1	3,4±0,2

lipids (lecithins and cephalins), sphingomyelins and inositol phospholipids, no definite changes could be observed within 2, 4, and 6 hours. However, within 24 hours after irradiation, while the total phospholipid content remained unchanged, the amount of the sphingomyelin fraction increased by 41.6%, and that of the lecithin fraction increased by 33.7%, with the cephalin fraction showing a 56.6% drop.

Evidently, during the first hours of irradiation, there take place in the animal organism powerful compensatory mechanisms, which keep the total phospholipid content within the normal range. The relative increase of the choline-containing fraction of phospholipids and lowering of its cephalin fraction within 24 hours after irradiation probably represent a corresponding change in the direction of the synthesis. According to the data of Elezarova [12], the amount of free choline in rat liver decreases, following irradiation of 700 r intensity. It is quite possible that the choline is here utilized for the intensive synthesis of choline-containing fractions of phospholipids.

The intensity of phospholipid exchange was judged by the rate of incorporation of labeled inorganic phosphorus. The incorporation of the latter was determined by the relative specific radioactivity (the ratio of the specific radioactivity of the phospholipid phosphorus to the specific radioactivity of inorganic phosphorus, multiplied by 100).

It can be seen from Table 2 that the intensity of renewal of the phosphate group in phospholipid, following irradiation, has markedly increased: The relative specific radioactivity of the total phospholipids has increased during the first four hours by 100%, after six hours by 137%, going slightly down after 24 hours, remaining 42% higher. The intensity of the lecithin and cephalin fractions underwent each a similar change. The relative specific radioactivity of the inositol phospholipids increased in two hours, after being subjected to radiation, by 60%, and during the remaining periods under study, by 120%. On the other hand, the relative specific radioactivity of the sphingomyelin fraction showed a tendency towards a decrease during the first four hours after irradiation, while after 24 hours one could observed a sharp increase in metabolism of this fraction, with the relative specific radioactivity raising 262.5% as compared with the control.

TABLE 2. Relative Specific Radioactivity of Total Phospholipids and Their Fractions in Rat Liver After Total Gamma-irradiation

Time after irradiation hr.	Total phospholipids in % of raw weight	Phospholipid fractions (in % of total)			
		lecithins	cephalins	inositol phosphatides	sphingomyelins
Control	2,4±0,1	2,4±0,1	2,3±0,1	1,5±0,1	0,8±0,1
2	4,8±0,4	4,5±0,4	4,4±0,3	2,4±0,3	0,6±0,1
4	4,5±0,4	4,2±0,5	4,1±0,5	3,3±0,2	0,6±0,1
6	5,7±0,3	5,4±0,3	4,8±0,4	3,2±0,2	1,9±0,3
24	3,4±0,1	3,2±0,1	3,2±0,1	3,1±0,3	2,9±0,2

Since the content of phospholipids and their fractions did not change during the first hours following irradiation, one may assume the gamma-ray irradiation is increasing specifically the renovation of the phosphoric portion of their molecules. Attention should also be called to the fact that within 24 hours following irradiation there is being observed a slight lowering in the renovation of lecithins and cephalins, where their fractions are being changed in opposite directions, the lecithins being on the increase and the cephalins falling. It seems that at this stage there actually takes place an increase of lecithin synthesis coupled with a breakdown of cephalins. Hokin and Hokin [13] observed the rate of renovation of the phosphate fraction of the pancreatic phospholipids in animals injected with acetylcholine. According to the authors, acetylcholine splits the molecules of lipoproteins, causing the liberation of phospholipids, which thus become more accessible to the action of the corresponding enzymes. Since the effect of ionizing

radiations can also result in a breakdown of lipoprotein complexes, it is possible that under these circumstances too an increase in phospholipid metabolism is tied to a higher vulnerability of their free molecules.

Where the intensity of the radiation is higher (the second series of experiments) there takes place an approximately 25% drop in the total phosphatides of the liver tissues, as early as within 10 minutes following irradiation (by a dose of 5000 r) (Table 3). A subsequent increase of the dose did not change this figure. As to the relative contents of separate phospholipid fractions, the changes were in general close to those observed in rats within 24 hours following irradiation with a dose of 1000r.

TABLE 3. Phospholipid Fractions in the Liver of Rabbits Immediately After Gamma-irradiation With Large Doses (in % of total phospholipids)

Irradiation dose, r	Phospholipid fractions			
	lecithins	cephalins	inositol phosphatides	sphingomyelins
	Controls			
—	67,2±0,6	17,2±0,6	1,1±0,2	2,1±0,1
	After irradiation			
5 000	76,3±1,3	11,9±0,4	0,8±0,1	2,7±0,3
15 000	72,1±1,3	14,3±0,8	1,1±0,2	3,5±0,3
30 000	74,9±1,0	16,4±0,5	1,2±0,2	3,5±0,3

Immediately after the beginning of irradiation, the relative amount of lecithins went slightly up: in 10 minutes, by 13.4%; subsequently, this higher level persisted. The cephalins dropped within 10 minutes by 34.7%. However, with radiation of longer duration and, correspondingly, at higher doses, the cephalins, while lowered, tend to approach normal values. The inositol phosphatides remained here practically unchanged. As to the sphingomyelins, they went up within 10 minutes, went still higher within 30 minutes, and stayed at the same level, 66.5% above normal, at 60 minutes.

Thus, under the influence of ionizing radiation, there occur in the liver tissue, in the course of the first minute and day, very great changes in the ratio between separate phospholipid fractions and their renovation as well, depending on the dose administered. This can be due to a certain extent to a breakdown of lipoprotein complexes and of the structure of cell organoids. On the other hand, such changes in the composition of the lipoproteins themselves will invariably lead to a change in their properties, as well as of the cell ultramicrostructures bound to these.

The breakup by phospholipases A and C of the 10% lecithin, which constitutes a part of succinoxidase, renders the performance of this enzyme system fully inactive [14]. Feeding animals a diet deficient in unsaturated fatty acids leads to a disruption in the metabolism of phospholipids, which form an integral part of the adenosine triphosphatase, and as a result, there takes place a disruption of oxidative phosphorylation and of the oxidation of intermediate products in the tricarbon cycle as well [15]. The activity of adenosine triphosphatase, which is activated by magnesium, and where phospholipids constitute the prosthetic group, drops in proportion to the activity of the phospholipases [16].

The lecithins participate in the transport of fatty acids in the organism, contribute to the oxidation of fatty acids, and stabilize the blood colloids. The cephalins play, evidently, an important role in the active transport of potassium and sodium cations across cell membranes. Therefore, the changes relating to the lecithin and cephalin content following irradiation may also cause disturbances in the specific functions of these phospholipids.

Of great importance is the "structural function" of phospholipids. We know today that sphingolipids are predominant in the exterior portions of cells, controlling permeability. Since sphingolipids are present in a number of tissues, one may assume a universal distribution of these substances in the animal organism and their great biolc ical importance. It was shown that in fat deficiency, changes are being observed in the composition of sphingolipids, consisting in a lowering in cerebrisides and an increase in sphingomyelin content. Changes in these important cell constituents result in a disturbance of the permeability of biological membranes [17]. The increased incorporation of P^{32} into the individual phospholipids following irradiation, is indicative of changes in phospholipid metabolism under the effect of radiation.

Hokin and Hokin [18] showed that the inositol phosphates are playing an important role in the secretion of protein and of other substances by endocrine glands. It is hard to say at this time what the role of inositol-phospholipids is relating to the function of internal-secretion glands and other organs, but the fact itself is of importance. In this respect, attention is being called

to the observed disturbance in inositol phosphatide metabolism of the liver, following irradiation.

SUMMARY

The total phospholipid content in rat liver does not change during the first day following irradiation by a 1000 r dose of gamma rays. In rabbits, on the other hand, the liver phospholipids immediately drop 25% subsequent to irradiation of not more than 5000 r.

In rat liver, the lecithin fraction of the phospholipids increases 33.7% within 24 hours following irradiation, the sphingomyelin fraction increasing under the same conditions 41.6%, while the cephalin fraction decreases by 56.6%. In rabbits, the lecithin fraction of the liver phospholipids rises 13.4% immediately following irradiation by 5000 r, the sphingomyelin fraction rising 66.5%, while the cephalin fraction drops 34.7%.

The general intensity of the phospholipid metabolism in rat liver increases within 24 hours following irradiation with a 1000 r dose. Within two and four hours the relative specific radioactivity of phospholipids increases 100%, within six hours it increases 137%, and within 24 hours, 42%. The incorporation of labeled phosphate into the liver sphingomyelins increases particularly in the 24 hours following irradiation; the relative specific radioactivity of this fraction is 262.5% above the control.

LITERATURE CITED

[1] E. F. Romantsev, in: Metabolism in Radiation Sickness [in Russian] by L. L. Ivanov, V. S. Balabukha, E.F. Romantsev, and T.A. Fedorova (Medgiz, Moscow, 1956), p. 139,

[2] C. Entenman and R. Neve, cited in: Problems of Radiobiology, ed. G.M. Frank [Russian translation] (IL, Moscow, 1956) p. 42.

[3] J.G. Coniglio, W.J. Darby, M.C. Wilkerson, et al., Amer. J. Physiol. 172, 86 (1953).

[4] V.D. Blokhina, Med. radiologiya 4, 1, 53 (1959).

[5] N.E. Ponomarenko, Report of the Scientific Conference on the Fortieth Anniversary of the Great October Socialist Revolution on the Problem: Pathogenesis, Clinical Practice, Therapy, and Prophylaxis of Radiation Sickness [in Russian] (Izd. Min. Zdravooch. SSSR, Leningrad, 1957), p. 30.

[6] H.P. Schwartz, H.E. Rigs, C. Glick et al., Proc. Soc. Exp. Biol. and Med. 80, 467 (1952).

[7] E.O. Weinman, S.K. Lerner, and C. Entenman, Arch. Biochem. and Biophys. 64, 164 (1956).

[8] S.V. Gasteva, O.V. Malinovskii, and D.A. Chetverikov, Report of the Scientific Conference on the Fortieth Anniversary of the Great October Socialist Revolution on the Problem: Pathogenesis, Clinical Practice, Therapy, and Prophylaxis of Radiation Sickness [in Russian] (Izd Min. Zdravooch. SSSR, Leningrad, 1957), p. 28.

[9] F.G. Sherman and A.B. Almeida, Adv Radiobiology 49 (1956).

[10] R.M.C. Dawson, Biochem. J. 57, 237 (1954).

[11] A.A. Smirnov and E.V. Chirkovskaya, Collection: Problems of the Biochemisty of the Nervous System [in Russian] (Izd. AN Ukr.SSSR, Kiev, 1957) p. 83.

[12] M.A. Eleazarova, Vestn. rentgenol. i. radiologii, 2, 22 (1956).

[13] M. Hokin and L. Hokin, J. Biol. Chem. 209, 549 (1954).

[14] S.W. Edwards and E.G. Ball, J. Biol. Chem 209, 619 (1954).

[15] P.D. Klein and K.M. Johnson, J. Biol. Chem. 211, 103 (1954).

[16] B.B. Kelley and O. Meyerhof, J. Biol. Chem. 183, 391 (1950).

[17] V.V. Nikol'skii, Dissertation: Origin and Role of High-Molecular Fatty Acids (C_{24}) in Animal Organisms [in Russian] (Rostov-on-Don, 1957).

[18] M. Hokin and L. Hokin, J. Biol. Chem. 233, 800 (1958).

THE BIOSYNTHESIS OF HIGHER ACIDS FROM ACETATE IN ISOLATED CHLOROPLASTS OF Spinacea oleracea LEAVES

B.P. Smirnov

Laboratory of Lipid Biochemistry, Institute of Biology,
Academy of Sciences USSR, Karelian Branch, Petrozavodsk
Translated from Biokhimiya Vol. 25, No. 3, pp. 545-555,
May-June, 1960
Original article submitted September 14, 1959

The study of biochemical processes in protoplasmic structures in vitro makes it possible to penetrate more deeply into the essential nature of cellular metabolism as a whole.

Investigations of fatty acid synthesis in plant cell organelles are extremely scanty. Sisakyan and Smirnov [1] have demonstrated acetate incorporation into higher fatty acids in isolated chloroplasts of sunflower cotyledons. Stumpf and Barber [2] have demonstrated synthesis of higher fatty acids by mitrochondria isolated from avocados; cofactors required for this synthesis are ATP, CoA, and Mn^{++}. Sunflower cotyledons and avocados are storage organs. The possibility of higher fatty acid synthesis by chloroplasts from leaves has not, however, been investigated.

This communication reports the synthesis of higher fatty acids from acetate by spinach leaf chloroplasts. Light has been shown to have an activating effect on this process. Cofactors required for acetate incorporation into higher fatty acids in both the dark and the light have been determined .

METHODS

Choroplasts were isolated from spinach leaves before the advent of the flowering stage. One hundred grams of leaves which had been thoroughly washed in distilled water were ground in a mortar with quartz sand in the presence of 80 ml 0.35 M sucrose made up in 0.2M tris buffer, pH 7.29. The homogenate was strained through linen and centrifuged twice for one-minute periods at 150g ts remove cellular debris. Chloroplasts were separated from the supernatant by centrifugation for 15 minutes at 900 g, and were then suspended in the buffered sucrose solution used earlier. Isolation of chloroplasts was carried out at 2°.

Labeled acetate, $CH_3C^{14}OOH$, and the necessary cofactors were added to the chloroplast suspension (total volume of the system,1.8 ml). Reactions mixtures were incubated in air in 50-ml Erlenmeyer flasks at 20-25° on a mechanical shaker at 50-60 strokes per minute for 30-120 minutes. Flasks of the "dark" group were enclosed in sacks of black material impermeable to light. Flasks of the "light" group were illuminated by a battery of six 500-watt electric lamps. A water bath of window glass 2 mm thick with a 2-cm layer of flowing water was interposed between the lights and the shaker in order to prevent heating of the chloroplast suspensions. Light intensity at the level of the flask was 7000 lux.

To inactivate the enzymes, flask contents were transferred to centrifuge tubes containing 2.5 ml of a saturated $NaHSO_4$ solution. The lipids were extracted with 1.0 ml chloroform by heating the tubes on a water bath at 40°, the tube contents being stirred with a glass rod. After centrifugation the chloroform extracts were separated off and a second extraction with a new portion of chloroform was performed. Chloroform extracts were placed on aluminum disks for determination of radioactivity in an MST-17 counter. Corrections were made for counter efficiency. A trial run showed that contaminating amounts of radioactive acetate not incorporated into the lipid fraction which may be present are completely evaporated from the disks in the course of a day.

After measurement of radioactivity, disks of a given treatment group from several experiments were pooled and extracted with anhydrous ethyl ether in a Soxhlet extractor. The percentage of total radioactivity of the lipid fraction ascribable to the higher fatty acid fraction was determined by two methods in parallel.

First method. Twenty ml of an ether extract of the lipid fraction was evaporated to a volume of 0.5 ml. In another tube, 0.2 g KOH was dissolved in 1.2 ml anhydrous glycerin by heating. The alkaline glycerin solution was poured into the tube containg the lipid fraction and heating was continued,with a vigorous stirring of the tube contents with a glass rod. The solution was cooled somewhat and then ~0.7 ml 25% H_2SO_4 was added by drops. The higher fatty acids, which had risen to the surface, were extracted three times with anhydrous ethyl ether. Radioactivity of the ether extracts was determined. From the data on radioactivity of the original lipid fraction and the radioactivity of the fatty acid component, the percentage of incorpo-

ration of acetate C^{14} into fatty acids was determined. Studies showed that ~93% of the acetate C^{14} incorporated into the lipids was associated with the fatty acid fraction; no substantial deviations from this distribution pattern were noted for any of the experimental treatments employed. For example, in one of the analyses original radioactivity of the lipid fraction was 3010 counts per minute, and radioactivity of the fatty acid fraction was 2820 counts per minute. Percentage of incorporation of acetate C^{14} into the fatty acid component of the lipid fraction was therefore 93.33.

Second method. Twenty ml of an ether extract of the lipid fraction was evaporated to a volume of 0.5 ml, 2 ml of 10% alcoholic KOH was added, and saponification was carried out by heating the mixture with a reflux condenser for 30 minutes. The aqueous extract of the salts of the fatty acids was neutralized with 2N H_2SO_4 and fractionated according to the method of Lehninger and Smith [3]. To 12.5 ml of a solution of neutralized fatty acids was added 1.0 ml 20% $CuSO_4$; the mixture was shaken and 1.5 ml of a 10% suspension of $Ca(OH)_2$ was added. The flask contents were stirred and centrifuged after 15 minutes. With such treatment, the fatty acids with more than ten carbon atoms form insoluble calcium and copper salts which precipitate. Radioactivity of the precipitate was determined, correction for absorption of β-rays by the precipitants being made. From the data obtained, the amount of radioactive acetate incorporated into the higher fatty acids ($> C_{10}$) was determined. Analysis of the distribution of label in the lipid fraction showed that ~75-80% of the radioactivity was concentrated in the higher fatty acids. No significant deviations from this pattern of distribution with experimental treatment were found. In addition to the fatty acids, acetate C^{14} could have been incorporated into the steroids of the lipid fraction. However, data on the radioactivity of the unsaponified part of the lipid fraction indicate that under the experimental conditions employed incorporation into steroids was slight in comparison with incorporation into higher fatty acids.

Calculation of incorporation of acetate into higher fatty acids of the lipid fraction was made according to the results on distribution of radioactivity with various experimental treatments obtained by the second method of fractionating the lipid extracts.

Nitrogen was determined by degradation of the chloroplast samples with H_2SO_4 in Kjeldahl flasks followed by determination of ammonia in Conway vessels [4]. Chlorophyll was determined by colorimetric measurements of alcoholic extracts of the chloroplast suspensions and comparison with standard chlorophyll solutions. Ratios between protein nitrogen content and chlorophyll content of the chloroplast suspensions were close to unity.

CoA preparations of 95% purity, Ba salt preparations of ADP, and AMP-5' preparations of 99% purity (L. Light firm) were employed. ADP was obtained from its Ba salt by the method of Umbreit, Burris, and Stauffer [5]. Results presented in Tables 1-3 and the two figures were obtained using an ATP preparation free of ADP and AMP-5'. The following experiments (Tables 4 and 5) were performed using an ATP preparation from the Ivanov meat plant. All acids were neutralized with potassium hydroxide to pH 7.25.

RESULTS

It was shown that during incubation of isolated chloroplasts with radioactive acetate in the dark there is very slight incorporation of acetate into the higher fatty acids. As Table 1 shows, the cofactors for this process are ATP, CoA, and Mn^{++}.

A study of the effect of length of incubation period and pH of the medium was made in order to determine optimal conditions for incorporation of acetate into the higher fatty acids. Fig. 1 shows that during the first 120

TABLE 1. Cofactors of Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts. (total reaction volume, 1.8 ml. incubation 60 minutes at 22° in the dark)

Reaction mixture components	Acetate incorporated, counts/min.
Complete system*	1,020
Complete system without ATP	120
" " " CoA	310
" " " $MnSO_4$	860
" " " ATP, CoA, $MnSO_4$	50

*Complete system contained 1.5 ml chloroplast suspension (15 mg protein) in 0.35M sucrose made up in 0.2M tris buffer, pH 7.20, and also (in μ moles): ATP—5.0; CoA—0.3; $MnSO_4$—0.9; labeled acetate—1.2 (0.5 μCurie).

minutes, amount of label incorporated is proportional to incubation time, after which incorporation slows down or ceases entirely. Incorporation is most effective at pH of 7.2-7.3 (Fig. 2). At concentrations of 0.6-1.2 μ moles per 1.8 ml incubation medium, labeled acetate did not inhibit synthesis of the higher fatty acids. At concentrations from $1.8 \cdot 10^{-5}$ to $1.8 \cdot 10^{-4}$, CoA stimulated fatty acid synthesis; higher concentrations were not studied. The presence of ATP in the reaction mixture is necessary for the effective incorporation of acetate into higher fatty acids. The optimal concentration is between $5.3 \cdot 10^{-3}$ and $10.6 \cdot 10^{-3}$ M (Table 2).

One of the cofactors of acetate incorporation into higher fatty acids is the Mn^{++} ion (Table 1). The effect of a number of concentrations of this ion on acetate incorporation is shown in Table 3; optimal levels were $1 \cdot 10^{-3}$ and $5 \cdot 10^{-4}$ M.

The data of Tables 1-3 indicate the existence of an enzyme system in spinach leaf chloroplasts which mediates the synthesis of higher fatty acids from acetate.

Incorporation of acetate into higher fatty acids involves the participation of CoA, which is a component of plant enzyme systems. Kaplan and Lipmann [6] found 0.74 units of CoA per g wet weight in spinach leaves. Our results (Table 1) indicate that addition of CoA to the incubation medium increases the rate of acetate incorporation into higher fatty acids, i.e., CoA is a coenzyme of acetylation in the chloroplasts. The role of acetic acid in fatty acid synthesis involves the formation of acetyl-CoA from acetate and CoA. The acetate-activating enzyme system which mediates this reaction was first found in plants by Millerd and Bonner [7]. The next stage involves the interaction of two acetyl-CoA molecules and the formation of acetoacetyl-CoA. By successive interactions with new acetyl-CoA molecules, the carbon chain of the fatty acid is build up. In their experiments with avocado mitochondria, Stumpf and Barber [2] have demonstrated the important role of ATP in fatty acid synthesis. Results presented in Tables 1 and 2 show that ATP is a necessary cofactor for the

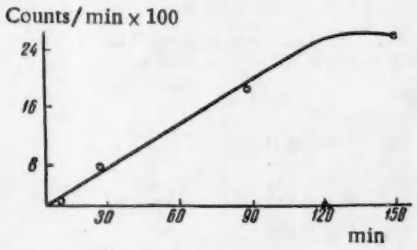


Fig. 1. The effect of length of incubation period on incorporation of acetate into higher fatty acids by spinach leaf chloroplasts. For experimental conditions, see Table 1.

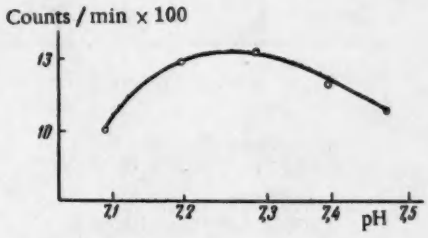


Fig. 2. The effect of pH of the medium on incorporation of acetate into higher fatty acids by spinach leaf chloroplasts. For experimental conditions, see Table 1.

TABLE 2. The Effect of ATP Concentration on Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts. Total reaction volume, 1.8 ml. gas phase, air. Incubation 95 minutes at 22° in the dark

Reaction mixture components	Acetate incorporated, counts/min.
Complete system*	440
Complete system + 5.0 μ moles ATP	1,140
" " +10.0 " "	2,110
" " +20.0 " "	4,480
" " +30.0 " "	2,720
Complete system without CoA, $MnSO_4$, ATP	90

*Complete system contained 1.5 ml of a chloroplast suspension (20 mg protein) in 0.35 M sucrose made up in 0.2 M tris buffer, pH 7.29, and also (in μ moles): CoA—0.3; $MnSO_4$—0.9; labeled acetate—1.2.

TABLE 3. The Effect of $MnSO_4$ Concentration on Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts. (for experimental conditions, see Table 2)

Reaction mixture components	Acetate incorporated, counts/min.
Complete system*	1 440
Complete system + 0.9 μ moles $MnSO_4$	1 800
The same + 1.8 μ moles	2 020
" " + 2.7 "	1 650

*Complete system contained 10 μ moles ATP; levels of other reaction components, except for $MnSO_4$, as in Table 2.

TABLE 4. The Effect of Illumination and of Cofactors on Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts (in counts/minute). (total reaction volume, 1.8 ml. Incubation 90 minutes at 22°)

Reaction mixture components	Conditions of incubation		Light / dark
	light	dark	
complete system*	8 240	2 370	3,5
complete system without ATP	2 220	360	6,2
" " " ATP, CoA, $MnSO_4$	660	240	2,8
complete system	10 560	3 330	3,2
complete system without ATP	1 870	390	3,8
" " " ATP, CoA, $MnSO_4$	390	60	6,5
complete system	13 660	3 120	4,4
complete system without ATP	3 360	630	5,4
" " " $MnSO_4$	11 800	2 860	4,2
" " " ATP, CoA, $MnSO_4$	490	105	4,7

*Complete system contained: 1.5 ml of a chloroplast suspension (~1.8 mg chlorophyll) in 0.4M sucrose made up in 0.2M tris buffer, pH 7.29, and also (in μ moles); ATP–5.0; CoA–0.3; $MnSO_4$–0.9; labeled acetate–1.2 (0.5 μCurie).

TABLE 5. The Effect of Illumination and ATP Concentration on Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts (in counts/minute).(total reaction volume, 1.8 ml. Incubation 90 minutes at 22°)

Reaction mixture components	Cond. of incubation		Light / dark
	light	dark	
Complete system	3 450	540	6,4
Complete system + 5,0 μmoles ATP	8 760	1 500	5,8
" " 10,0 " "	10 290	2 790	3,7
" " 20,0 " "	13 020	6 000	2,1
Complete system without CoA, $MnSO_4$, ATP	500	120	4,2

*Components of the complete system, except for ATP, as in Table 4.

enzymatic incorporation of acetate into higher fatty acids by spinach leaves in the dark. The mechanism of activation by Mn^{++} ions (Tables 1 and 3) is still unclear.

Later studies were devoted to the effect of illumination on higher fatty acid synthesis. Table 4 shows that the rate of acetate incorporation by chloroplasts is increased by illumination.

As Table 4 shows, cofactors for the photosynthetic incorporation of acetate into higher fatty acids are CoA, Mn^{++}, and the ATP preparation. Differences between incorporation in the light and the dark were observed both with the complete system and also with systems from which one or all the cofactors had been excluded, although in this case the rate of photosynthetic incorporation is reduced several-fold (Table 4). With an increase of ATP concentration from 5 to 20 μ moles in the incubation mixture, incorporation rate increases (Table 5).

Table 5 shows that as ATP concentration is increased the ratio between incorporation in the light and in the dark (light/dark) falls. Thus, at an ATP concentration of 5 μ moles it is 5.8, while at a concentration of 20 μ moles it is 1.2. Therefore, an increase in ATP has resulted in an increase of acetate incorporation at the expense of the high-energy bond of ATP and to a reduction in the relative rate of photosynthetic incorporation. An analysis of the data of Tables 4 and 5 also shows that the highest light/dark ratios were obtained in the absence of ATP alone or of ATP together with other factors. This led to the hypothesis that photosynthetic incorporation is not dependent on ATP. The amount of acetate incorporated in the light in the absence of ATP is, however, slight. Addition of ATP to the chloroplast suspension resulted in an increase in the amount of acetate incorporated photosynthetically. This led us to assume that the ATP preparation used also contained other nucleotide which probably participate in the photosynthetic incorporation of acetate into higher fatty acids. Analysis of the ATP preparation used in the experiments reported in Tables 4 and 5 revealed that they contain 69% ATP, 23% ADP, and 5% AMP. (Determinations were made with the anion exchange resin EDE-10-P[8]). Thus, the assumption developed that possibly ADP and AMP-5' participate in the photosynthetic incorporation of acetate into higher fatty acids by chloroplasts.

Results of studies of the effect of ADP, AMP-5' and inorganic P on the photosynthetic incorporation of acetate are presented in Table 6.

These results indicate that ADP is a cofactor of acetate incorporation into higher fatty acids. Addition of ADP to the incubation mixture resulted in a significant increase in rate of label incorporation in the light and a slight increase in the dark. The light/dark ratio in the presence of ADP was always increased over the control (system without ADP).

Addition of P_{inorg} together with ADP (Table 6) depressed incorporation. P_{inorg} was added to the incubation medium (together with ADP) in order to clarify the relation between the photosynthetic acetylation found in this study and photosynthetic phosphorylation which occurs in chloroplasts [9-11]. Molar concentrations of ADP and P_{inorg} corresponded to those necessary for photosynthetic phosphorylation in suspensions of spinach chloroplasts.

TABLE 6. The Effect of ADP, AMP-5', and Inorganic Phosphate on the Photosynthetic Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts (in counts/minute). (Total reaction volume 1.8 ml. Incubation 120 minutes at 22°)

Reaction mixture components, μ moles	Conditions of incubation		Light
	light	dark	dark
Complete system	3 600	600	6,0
Complete system + 4.1 ADP	8 280	900	9,2
" " + 4.1 ADP and 5.0 KH_2PO_4	3 120	330	9,5
Complete system	3 500	560	6,3
Complete system + 4.1 ADP	8 160	960	8,5
" " + 4.1 ADP and 5.0 KH_2PO_4	4 680	480	9,8
Complete system	2 240	520	4,3
Complete system + 4.1 ADP	5 900	870	6,8
" " + 4.1 ADP and 3.7KH_2PO_4,Na_2HPO_4	4 700	990	4,7
" " + 7.3 AMP	1 900	780	2,4
" " + 7.3 AMP and 3.7KH_2PO_4,Na_2HPO_4	1 530	870	1,8
Complete system	3 390	630	5,4
Complete system + 4.1 ADP	6 190	810	6,4
" " + 4.1 ADP and 3.7KH_2PO_4,Na_2HPO_4	4 920	920	5,3
" " + 7.3 AMP	1 950	780	2,5
" " + 7.3 AMP and 3.7KH_2PO_4,Na_2HPO_4	1 530	900	1,7
Complete system	920	120	7,7
Complete system + 4.1 ADP	6 600	240	27,5
" " + 4.1 AMP	660	90	7,3

*Components of complete system, except for ATP, as in Table 4. KH_2PO_4 and Na_2HPO_4 introduced as a solution buffered at pH 7.29.

TABLE 7. The Effect of ADP Concentration on Photosynthetic Incorporation of Acetate into Higher Fatty Acids by Spinach Leaf Chloroplasts (in counts/minute). (total reaction volume, 1.8 ml. Incubation 120 minutes at 22°)

Reaction mixture components in μ moles	Cond. of incubation		Light
	light	dark	dark
Complete system *	2 040	300	6,8
Complete system + 3.6 μ moles ADP	7 980	600	13,3
" " + 1.8 " "	7 560	570	13,3
" " + 0.9 " "	5 400	420	12,9

*Components of complete systems, except for ATP, as in Table 4.

It could be concluded on the basis of these data (Table 6) that photosynthetic incorporation of acetate into higher fatty acids is not related to the photosynthetic formation of ATP. When ADP and P_{inorg} were present, photosynthetic formation of ATP could take place, and yet, as the results show, P_{inorg} depressed photosynthetic incorporation of acetate.

It should also be noted that photosynthetic phosphorylation of ADP in chloroplasts from young spinach leaves proceeds only in the presence of ascorbic acid [12]; no ascorbic acid was added to the incubation mixture in our experiments, however, and nevertheless photosynthetic incorporation of acetate was vigorous. This is still further proof that in these experiments there was photosynthetic incorporation of acetate into higher fatty acids involving ADP; such incorporation was not related to the photosynthetic formation of ATP and a subsequent utilization of its energy.

When AMP-5' was added to the incubation medium (Table 6), photosynthetic incorporation of the label was reduced. The reduction was still greater if $P_{inorg.}$ was added together with AMP-5'. Concentrations of AMP-5' and P_{inorg} employed were those required for the photosynthetic phosphorylation of adenylic acid in chloroplast suspensions which has previously been thought to occur [9,10]. These results indicate that AMP-5' does not participate in photosynthetic acetylation under the experimental conditions employed.

Experiments on photosynthetic acelylation with ATP preparations completely free of admixtures of ADP were not performed, since ATPase is very active in spinach chloroplasts [13]. Such experiments would therefore be of little value without the use of a specific inhibitor of ATPase.

In the light of the discovery of participation of ADP in photosynthetic acetylation, the effect of various ADP concentrations on rate of acetate incorporation was then studied (Table 7).

It was established that catalytic amounts of ADP (0.9 μ mole/sample) are required for photosynthetic acetylation; a four-fold increase in ADP results in only a slight increase in the light/dark ratio.

TABLE 8. The Dependence of Photosynthetic Incorporation of Acetate (in counts/minute) into Higher Fatty Acids on Chlorophyll Content of the Chloroplast Suspension. System Contained: 1.5 ml of a Chloroplast Suspension in 0.4M Sucrose made up in 0.2M Tris Buffer, pH 7.29, and also (in μ moles): CoA—0.3; ADP—2.0; $MnSO_4$—0.9; C^{14}-acetate—1.2 (0.5μ Curie). (total reaction volume, 1.8 ml. Area of the base of the erlenmeyer flasks in which incubation was carried out—16 cm_2. Light intensity, 7000 Lux. Incubation 120 min. at 20°)

Chlorophyl content of chloroplast suspension, mg	Cond. of incubation		Light
	light	dark	dark
3,4	8 430	1 590	5,3
1,7	8 670	1 560	5,6
0,8	9 000	1 350	6,7
0,4	4 560	600	7,6
0,2	1 770	480	3,7

Results of all the experiments reported in Tables 6 and 7 are in close agreement as to the absolute amount of acetate incorporated into higher fatty acids in the light in the presence of ADP under identical experimental conditions. The relatively high ratio in one of the experiments with ADP (Table 6) is due to the small dark incorporation, which is evidently the result of a reduced ATP level in the chloroplasts, a condition associated with the state of the leaves prior to extraction of the chloroplasts (light conditions, temperature conditions, etc.).

The rate of photosynthetic incorporation of acetate into higher fatty acids depends, as was to be expected, on the amount of chlorophyll in the chloroplast suspension (Table 8).

The highest rate of incorporation was observed at a total chlorophyll content of ~0.8 mg. Further increase in the quantity of chloroplasts in the suspension did not result in an increased incorporation rate; this is evidently associated with an insufficient illumination throughout the chloroplast suspension.

TABLE 9. The Effect of Thorough Washing of Chloroplasts on Photosynthetic Incorporation of Acetate into Higher Fatty Acids (counts/minute).(total reaction volume, 1.8 ml. Incubation 120 minutes at 22°)

Reaction mixture components	Cond. of incubation		Light
	light	dark	dark
Unwashed chloroplasts			
Complete system *	960	320	3,0
Complete system + ADP	5 520	570	9,7
Complete system + ADP, KH_2PO_4, Na_2HPO_4	3 330	540	6,2
Complete system + CoA, $MnSO_4$	660	240	2,8
Washed chloroplasts			
Complete system	420	240	1,8
Complete system + ADP	1 420	390	3,6
" " + ADP, KH_2PO_4, Na_2HPO_4	990	460	2,2
" " without CoA, $MnSO_4$	360	120	3,0

*Complete system contained: 1.5 ml of a chloroplast suspension (1.5 mg chlorophyll) in 0.4M sucrose made up in 0.2 M tris buffer, pH 7.29, and also (in μ moles) CoA – 0.3; $MnSO_4$ – 0.9; C^{14}-acetate – 1.2 (0.5 μ Curie); ADP – 3.6; KH_2PO_4 3.7 (buffer pH 7.29).

When chloroplasts were washed in the sucrose buffer solution two times, there was a significant decline in the rate of acetate incorporation under comparable experimental conditions. In the experiment summarized in Table 9, the isolated chloroplasts were suspended in 50 ml of 0.4M sucrose made up in 0.2M tris buffer, pH 7.29, and then precipitated by centrifugation at 900 g for 10 minutes at 2-4°. This washing procedure was then repeated. Experiments were carried out with chloroplast suspensions containing the same amounts of chlorophyll per unit volume.

Table 9 shows that when chloroplasts are washed twice there is a reduction in the rate of photosynthetic incorporation of acetate into higher fatty acids and also a reduction in dark incorporation rate which is relatively smaller. The activation of acetate incorporation by ADP is observed both in the light and the dark. The nature of the activating factor which is removed as a result of washing of washing is still obscure.

Plant age is of considerable influence on the rate of photosynthetic acetate incorporation. The highest rates were obtained with chloroplasts isolated from leaves prior to the advent of flowering. They were somewhat lower during the flowering stage, and during the seed-maturation stage no incorporation was found.

This may be explained as follows. At early stages of development there is a rapid formation of chloroplasts in leaf cells. The utilization of light energy for the photosynthetic formation of higher fatty acids promotes an intensification of synthetic processes in chlorophyllous tissues. At later stages of development, formation of protoplasmic structures slows down, and since the higher fatty acids are not transportable products of photosynthesis, their photosynthetic formation slows down, and later ceases entirely.

DISCUSSION

The data presented indicate two different mechanisms of acetate incorporation in fatty acid biosynthesis by spinach leaf chloroplasts. One involves the utilization of the high-energy bond of ATP, while the other is based on the more direct utilization of light energy.

A group of American workers under Arnon [14] has shown that the transformation of light energy into energy of ATP occurs in chloroplasts according to the following scheme:

$$ADP + P_{inorg} \xrightarrow[\text{chlorophyll}]{\text{light}} ATP$$

Our data have shown that the photosynthetic incorporation of acetate into higher fatty acids is not energetically related to the photosynthetic formation of ATP.

The following mechanism of acetate activation, one which assumes an intermediate acetyl-adenylate compound (acetyl ~ AMP), was established by Berg [15, 16] for heterotrophic organisms (yeasts).

$$ATP + \text{acetate} \rightleftarrows ATP\sim\text{acetyl} + PP$$

$$AMP\sim\text{acetyl} + CoA \rightleftarrows \text{acetyl}\sim CoA + AMP$$

Berg's conclusions have been confirmed by studies with microorganisms [17, 18] and by experiments with pigeon liver enzyme preparations [19].

The cofactors of photosynthetic incorporation of acetate into higher fatty acids in our investigations

are ADP, CoA, and Mn^{++} ions; the process requires light and chlorophyll. AMP and P_{inorg} inhibit the reaction. The following scheme for photosynthetic incorporation of acetate into higher fatty acids is proposed on the basis of the data presented:

$$\text{ADP} + \text{acetate} \xrightarrow[\text{chlorophyll}]{\text{light}} \text{ADP}\sim\text{acetyl}$$

$$\text{ADP}\sim\text{acetyl} + \text{CoA} \longrightarrow \text{acetyl}\sim\text{CoA} + \text{ADP}$$

A high-energy ADP~acetyl compound is formed in the light in the presence of chlorophyll and then reacts with CoA to give acetyl-CoA. The carbon chain of the highe fatty acids may then be built up by successive interactions of several acetyl-CoA molecules. ADP acts as an acetate acceptor in photosynthetic acetylation. In the transfer of the acetyl residue from ADP~acetyl to CoA, there is a reduction of the ADP molecule. Thus, while during photosynthetic phosphorylation in chloroplasts [14] there is an uptake of $P_{inorg.}$ by ADP, during photosynthetic acetylation there is an uptake of acetate by ADP; in each case, a high-energy compound is formed at the expense of light energy. The scheme of photosynthetic activation of acetate proposed requires more intensive study, and further investigations should be directed primarily to the establsihment of the as yet hypothetical intermediate ADP~acetyl.

Acetate incorporation in the dark utilizes the energy of ATP, other cofactors being CoA and Mn^{++} ions.

The mechanism of acetate incorporation in the dark, which is derived from that of incorporation in the light, may be indicated as follows:

$$\text{ATP} + \text{acetate} \longrightarrow \text{ADP}\sim\text{acetyl} + \text{P}$$

$$\text{ADP}\sim\text{acetyl} + \text{CoA} \longrightarrow \text{acetyl}\sim\text{CoA} + \text{ADP}$$

ADP~acetyl is formed by the interaction of acetate and ATP. The following reaction (formation of acetyl--CoA) is the same as for photosynthetic incorporation.

The role of Mn^{++} ions probably involves formation of complexes of Mn^{++} with certain intermediates during acetate activation, which lowers the energy barriers to reactions involving with intermediates.

The biological significance of the mechanism of photosynthetic acetylation proposed is that with the formation of ADP~acetyl at the expense of light energy an additional energy transfer, from ATP, is eliminated; the system is thus more advantageous from an energetic point of view, since energy transfers through additional reactants lead to unproductive energy losses.

SUMMARY

The in vitro incorporation of 1-C^{14} acetate into higher fatty acids of chloroplast lipids in spinach proceeds in two different ways.

The cofactors of dark incorporation of acetate into higher fatty acids of the chloroplast lipids are ATP, coenzyme A and Mn++ ions.

The cofactors of photosynthetic incorporation of acetate into higher fattty acids of chloroplast lipids are ADP, coenzyme A and Mn^{++} ions. The process occurs at the expense of light energy with the participation of chlorophyll. Inorganic phosphorus and adenylic acid inhibit photosynthetic acetylation.

A scheme is presented illustrating photosynthetic activation of acetate in plant chloroplasts.

LITERATURE CITED

[1] N.M. Sisakyan and B.P. Smirnov, Biokhimiya 21, 273 (1956).*

[2] P.K. Stumpf and C.A. Barber, J. Biol. Chem 227, 408 (1957).

[3] A.L. Lehninger and S.W. Smith, Biol. Chem. 173, 773 (1948).

[4] N.P. Meshöva and S.E. Severin. Manual of Animal Biochemistry [in Russian] (ISN, Moscow, 1950).

[5] R.H. Umbreit and J.F. Stauffer. Manometric Techniques and the Study of Tissue Metabolism. [Russian translation] (IL, Moscow, 1951).

[6] N.O. Kaplan and F. Lipmann, J. Biol. Chem. 174 37 (1948).

[7] A. Millerd and J. Bonner, Arch. Biochem. and Biophys. 49, 343 (1954).

[8] A.V. Kotel'nikova, E.L. Dovedova, and V.V. Solomatina, Biokhimiya 24, 215 (1959).*

[9] D.L. Arnon, M.B. Allen and F.R. Whatley, Nature 174, 394 (1954).

[10] M.B. Allen, D.I. Arnon, J.B. Capidale, et. al., J. Am. Chem. Soc. 77, 4149 (1955).

[11] A.V. Trebst, H.Y. Tsyjimoto and D.I. Arnon, Nature 182, 351 (1958).

[12] W. Fuchtbauer and W. Simonis, Naturw. 21, 519 (1958).

[13] H. Baltsheffsky, Acta chem. scand. 13, 393 (1959).

[14] D.I. Arnon, in: Photochemical Apparatus, its Structure and Function 11, p. 181, Brookhaven symposia in Biology (1958).

[15] P. Berg, J. Am. Chem. Soc. 77, 3163 (1955).

[16] P. Berg, J. Biol. Chem. 222, 1025 (1959).

[17] M.A. Eisenberg, Biochim. et biophys. acta 16, 58 (1957).

[18] M.A. Eisenberg, Biochim. et biophys. acta 23, 317 (1957).

[19] M. Whitehouse, H. Moeksi, and S. Curin, J. Biol. Chem. 226, 813 (1957).

*Original Russian pagination. See C.B. Translation.

THE EFFECT OF INSULIN ON GLYCOLYSIS IN TISSUE CULTURE CELLS

R.N. Étingof and A.A. Krichevskaya

Biochemical Laboratory, Institute of Poliomyelitis Study, Academy of Medical Sciences USSR, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 556-562, May-June, 1960

Origimal article submitted September 16, 1959

It was shown in previous communications [1, 2] that various tissue cultures possess common metabolic characteristics, such as dislocations in the respiratory chain and a high level of aerobic and anaerobic glycolysis, which are independent of their origin (normal and malignant cells), the DNA content in the nucleus (diploidal and heteroploidal arrangement of chromosomes), and the duration of their stay in vitro. As evidenced by a number of available data [3-5], anaerobic glycolysis is of basic importance for the life function of tissue cultures and the reproduction therein of a series of viruses. Therefore, the possibility of changes in this very process should be of essential interest.

A study of the influence of individual hormones on cell cultures presents a definite interest also when viewed as an attempt of selective regulation of metabolism on the cell level. As to insulin, it is known that this hormone affects certain metabolic processes in a number of tissue cultures, glycolysis in particular [6, 7]. In addition, the activation of glycolysis in the cells of malignant origin, as contrasted with embryonic cells which are resistant to insulin, is considered by some authors as one of the potential criteria of cells turning malignant under in vitro conditions [6]. In the light of these, as well as some other published data [8, 9], it is evident that tissue cultures offer a convenient model for the study of a number of problems linked with the elucidation of hormonal effect on isolated processes in whole uninjured cells.

The subject of the present communication consists of results obtained by us in the study on the effect of insulin on the glycolysis of various cell cultures.

EXPERIMENTAL

Origin of cell material. The work was conducted on the following cell cultures: Seven–ten days old cultures of monkey (Macacus rhesus) kidney cortex (MK); cells from cancer cultures NEC-2 and HeLa [10, 11], originating from malignant degenerated human epithelium; cells isolated from monkey heart (Macacus cynomolgus) (MH) [12], and cells obtained from embryonic rabbit kidney (REK) [13]. With the exception of MK, all of the enumerated cultures were continuous, namely, being regularly transplanted.

The methods used in trypsin treatment, in culture technique, as well as for the removal of cells from the glass surface and their count, are described in previous communications [1, 2, 14]. Synthetic mixture No. 199, containing 20% human serum, served as nutrient medium for the HeLa cells.

Biochemical methods. The manometric measurements and determinations of glucose and lactic acid were conducted as described in a previous work [2]; in samples containing sucrose, the conditions for the precipitation of carbohydrates were somewhat modified to permit the determination of lactic acid; the fractionation techniques have already been described [14]. A somewhat modified Tiedemann's medium [15] was used for the determination of the glycolytic activity of separate cell fractions. The intensity of glycolysis was expressed in μg of lactic acid (accumulated within one hour of incubation at 37°) per 10^6 nuclei or per 1 mg of nitrogen determined colorimetrically by means of Nessler's reagent. The statistical processing of the results was conducted in the usual way [16]. Various types of insulin were used: a domestic crystalline preparation, and in a few experiments in soluble form, as put out by Novo firm.

RESULTS AND DISCUSSION

Effect of Insulin on Glyolysis in Whole Cells

In this series of experiments tests were performed on the effect of insulin on the intensity of glycolysis in various tissue cultures during proliferation and the stationary stage as well.

In experiments with proliferating cells, insulin was introduced into the culture medium (synthetic mix No. 199) in amounts of 0.1 units per ml, and after an interval of 24 hours, glucose and lactic acid were determined in the fluid portion, as well as in the controls, of which every experiment contained not less than six. The increment index of the cell growth was determined from the ratio of the initial number of cells introduced into the medium and their number after a 24-hr incubation period. The mean of the number of cells present in the medium at the start and after 24 hours was

taken as their average number during this period. The experiments with cell cultures in the stationary state were conducted on cell suspensions in medium No. 199 (without serum) for a two-hour period.

TABLE 1. The Effect of Insulin on the Utilization of Glucose and the Accumulation of Lactic Acid in the Process of Proliferation of Tissue Cultures. (the figures represent the result of statistical processing of the percentage changes in the amount of glucose used and lactic acid formed in samples containing insulin, as compared with the controls

Culture	Glucose	Lactic acid
MH	$+39,7 \pm 8,4$* $t = 4,8$; $p < 0,01$; $n = 8$	$+43,0 \pm 8,5$ $t = 5,1$; $p < 0,001$; $n = 9$
NEC-2	$+12,9 \pm 3,3$ $t = 3,9$; $p < 0,01$; $n = 9$	$+12,8 \pm 4,0$ $t = 3,3$; $p < 0,01$; $n = 10$
MK	$+5,7 \pm 2,7$ $t = 2,1$; $p > 0,05$; $n = 6$	$+3,0 \pm 3,6$ $t = 0,8$; $p > 0,05$; $n = 6$

*In this table is indicated the mean with mean deviation, giving the essence of the difference (t) and the number of experiments (n).

TABLE 2. Effect of Insulin on the Cell Mass Increment

Culture	Increment index*	
	without insulin	with insulin
MH	1,47 12 expts.	1,48 9 expts.
NEC-2	1,60 9 »	1,63 8 »
MK	1,35 6 »	1,35 6 »

*The ratio between the final cell count to the initial one.

Table 2 shows the results of the experiments relating to the influence of insulin on the glycogen in cells, MH, NEC-2 and MK. It is evident that insulin causes in MH cells a significant increase (average 40%) in the utilization of glucose and an accumulation of lactic acid; less distinct, but quite definite statistically, is the effect of insulin on glycolysis in cells NEC-2. As to cells MK, the results are so far statistically uncertain, although in a few experiments there takes place an activation of glycolysis under the effect of insulin. It should be noted that according to some published data [6, 17, 18], insulin is capable of stimulating the mitotic activity of cells, which, according to Swann's concept [19], may be one of the reasons for a stimulation of the utilization of glucose. However, under our experimental conditions, insulin did not affect the accumulation of the cellular mass. The increment index in all of the cultures under study remained constant, whether in the presence or absence of insulin (Table 2).

Table 3 gives the results of experiments dealing with the effect of insulin on the accumulation of lactic acid by various cell cultures in a stationary state. The results are analogous with the ones obtained in the experiments with proliferating cells. Insulin did stimulate the accumulation of lactic acid by all cell cultures, except the MK cells. The comparatively high sensitivity of MH cells to the glycolytic effect of insulin is to be noted. In this instance, too, the activation amounted to about 40% (Table 1). In all of the other cultures the increase in glycolysis amounted to about 25%.

TABLE 3. Effect of Insulin on Lactic Acid Accumulation in a Stationary Culture. (designations as in Table 1)

Culture	Lactic acid
MH	$+43,8 \pm 9,3$ $t = 4,7$; $p < 0,001$; $n = 14$
NEC-2	$+25,0 \pm 4,7$ $t = 5,3$; $p < 0,001$; $n = 8$
MK	$+0,4 \pm 5,8$ $t = 0,07$; $p > 0,05$; $n = 7$
REK	$+26,2 \pm 8,2$ $t = 3,2$; $p < 0,01$; $n = 4$
HeLa	$+28,0 \pm 8,3$ $t = 3,4$; $p < 0,01$; $n = 4$

It is evident from the obtained results that the glycolysis in all of our continuous samples, derived from tumors (the HeLa and NEC-2 cells) as well as from normal tissues (MH and REK cells), was equally sensitive to insulin. It is of interest that, in respect to DNA phosphorus content in the nucleus (which corresponds to the chromosome accumulation), the NEC-2, MH, and REK cells are approximately equal [2, 13]. The glycolysis in the cells of the original culture MK is resistant to insulin; the amount of DNA-phosphorus in the nucleus of these cells corresponds to a normal, diploidal assortment of chromosomes. It appears very tempting to venture into the correlation between sensitivity to hormonal influences and the gradual transformation of the chromosome apparatus of the cell. However, any conclusions in this plane will be premature; one should take into consideration the fact that in primary cultures there may still be some antiinsulin forces at work. But one should note that after the initial "jump", e.g., sharp metabolic changes immediately following the transplantation of the tissue into in vitro conditions, there is being observed, in the course of a prolonged culture, also a subsequent metabolism transformation [20, 21]. From this point of view it is of great interest that a number of

viruses are well multiplying in primary cultures, while adapting themselves with difficulty to continuous tissue cultures.

As to the sensitivity of glycolysis to insulin as a criterion of malignancy, it can hardly be depended upon, as already pointed out by us [2]. The difference in sensitivity to insulin glycolysis by embryonic and tumor cells observed by Leslie [6] can, presumably, be explained by the differences in the period of stay, under in vitro conditions. It is not clear how to select the crucial point in the life process of a culture where the cell ceases to be normal and becomes cancerous.

It is quite unlikely that the mechanism of insulin action on cell glycolysis in tissue cultures could be linked to an increased penetration of glucose into the cells, namely, permeability. According to Agol [22], the permeability of MH cells to glucose is very high; besides, in some of our experiments insulin was capable of inducing also a so-called "endogenous" glycolysis, e.g., an accumulation of lactic acid at the expense of intracellular stored materials. One should rather presume the possibility of the effect of insulin on some enzyme systems [23]. The fact stands out that , with a few exceptions, there exists a direct relationship between the utilization of glucose and the accumulation of lactic acid. It probably indicates that the primary effect of insulin is linked with glucose transformation [24].

Glycolysis of the Mitochondria of Monkey Heart Cells and the Effect of Insulin

The problem of the presence in mitochondria of a glycolytic setup of enzymes is being answered in different ways for mitochondria of different origin. Contrary to the mitochondria of the liver and kidneys, the mitochondria of brain and certain tumors possess glycolytic capacity [25, 26]. In view of the similarity in certain metabolic aspects of tissue cultures and malignant tissues, one could expect that the mitochondria of tissue cultures would also possess glycolytic capacity.

Figure 1 represents the results of a typical experiment dealing with the determination of glycolytic activity of separate cell fractions, expressed in μg of the lactic acid formed per mg N. As to the nuclei, this fraction was not of sufficient purity (it contained too many cell fractions), which prevented the determination of its activity. The most intensive glycolyzing activity was exhibited by the supernatant fraction, where the glycolytic activity was not less, but equalto, and even higher than the one of the issuing homogenate, contrary to the classical data of Schneider and Potter [27] on liver. As a rule, the mitochondria possess glycolytic activity, which is,however, insignificant, constituting one-sixth of the combined glycolytic activity of the homogenate. Thus, in this respect the mitochondria of tissue cultures remind one of the mitochondria of brain and tumors rather than of liver and kidneys. In a number of instances the glycolytic system of the mitochondria could be substantially activated by the addition of boiled supernatant fraction, which indicates a lack of some thermostable cofactors (Fig. 2). It is of interest, that the lower the issuing glycolytic activity of the mitochondria as such, the higher the degree of activation (experiments 1 and 2), and,in the instances where the issuing glycolytic activity is comparatively high, no activation actually takes place on the addition of boiled supernatant fraction (experiments 3 and 4). Activation of mitochondrial glycolysis by boiled supernatant fraction was observed also on brain and tumor mitochondria [25], which confirms the similarity of certain phases of their metabolism.

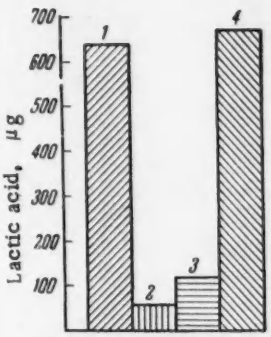


Fig. 1. Glycolysis in the separate fractions of MH cells. The height of the column corresponds to the amount of accumulated lactic acid in μg/mg N per hr at 37°C. 1, the homogenate; 2, the nuclei; 3, the mitochondria; 4) the supernatant fraction

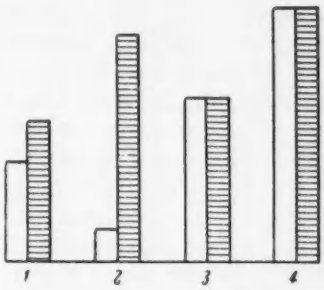


Fig. 2. Effect of boiled supernatant fluid on mitochondria glycolysis. 1,2,3,4, the experimental numbers. The light columns represent the mitochondria; the shaded columns,- the mitochondria+the supernatant fraction.

TABLE 4. Effect of Insulin on the Glycolysis of Mitochondria of Monkey Heart Cells. (amount of glycolysis in mg lactic acid/mg N)

Expt. No.	Mitochindria	Mitochindria + insulin	Changes %
1	22*	25	+ 14
2	84	84	0
3	63	63	0
4	71	69	— 3
	71**	69**	— 3
5	112	106	— 5
	112**	106**	— 5
6	39*	34	— 13

*Mitochondria without the addition of boiled supernatent fluid.

**The experiments were conducted under anaerobic conditions.

The difference in activating potency of the supernatent fraction, which was observed in some experiments, could perhaps be explained by a different degree of mitochondrial rinse, while the latter was invariably performed in the same way, no less than twice. It is, however, not excluded that in individual experiments we were dealing with somewhat different mitochondria, since it is known from morphology [28] that the mitochondria of tissue cultures are quite labile.

Table 4 represents the experimental results on the effect of insulin (2 units per 2 ml of media) on glycolysis in mitochondria under various conditions. The results show, in this case, the absense of insulin effect. In this respect, our data differ from those published by Hochstein [29], who observed an activation of glycolysis by insulin by the mitochondria of ascitic tumors. One should, however, point out that Hochstein's experiments were conducted at 20°C for a three -hour period. It is known, that the temperature factor plays an essential role in hormonal influence [30]. Thus, the "structurally organized" glycolysis under our experimental conditions proved to be resistant to insulin.

In conclusion, one should point out that a study on the influence of insulin on some unorganized systems of tissue cultures seems very promising, as far as these cells are "freed" from any regulatory agents, due to the peculiar conditions of their existence, and the effect of separate factors can be manifested more clearly. As to insulin, it is capable not only of influencing the structure of the protein molecule [31], but also of affecting the activity of some purified enzyme systems [23, 32, 33]. There are good reasons to assume that there is a definite relationship between these factors.

SUMMARY

Insulin (0.1 unit per ml media) boosts the utilization of glucose and the accumulation of lactic acid in the process of proliferation of continuous tissue culture specimen (the cancerous NEC-2 and the sample of cells derived from monkey heart). When applied in the same concentrations, insulin activates the accumulation of lactic acid also where the culture of these specimens is in a stationary state, as well as in the cancerous HeLa specimen and in cells derived from the embryonic kidney of rabbit. The glycolysis of a primary culture (the cortex of monkey kidney) remains under these conditions resistant to insulin.

The main portion of glycolytic activity of a cell homogenate, derived from a monkey heart, is concentrated in the supernatant fraction, but the mitochondria of these cells also possess glycolytic capacity. In some instances, the glycolytic ability of the mitochondria can be substantially activated by the addition of the boiled supernatant fraction. The mitochondrial glycolysis is insensitive to insulin.

The obtained data were discussed from the point of view of the peculiarity of tissue culture metabolism and of the mechanism of insulin action.

LITERATURE CITED

[1] V.N. Gershanovich, V. I. Agol, R. N. Etingof, and S.G. Dzagurov, Biokhimiya 23, 453 (1958).*

[2] V.I. Agol, V.N. Gershanovich, and R.N. Étingof, Biokhimiya 24, 101 (1959).*

[3] O. Warburg, Science 123, 309 (1956).

[4] J. Jones and S.L. Bonting, Exptl. Cell Res. 10, 631 (1956).

[5] J. Becker, N. Grossowicz, and H. Bernkopf, Proc. Soc. Exp. Biol. and Med. 97, 77 (1958).

[6] J. Leslie, W.C. Fulton, and R. Sinclair, Biochim. et biophys. acta 24, 365 (1957).

[7] S. Graff and K.S. McCarty, Cancer Res. 18, 741 (1958).

[8] H. Grossfeld, Science 127, 148 (1958).

[9] S. Halevy and L. Avivi, Biochim. et biophys. acta 30, 198 (1958).

[10] H.W. Toolan, Cancer Res. 14, 660 (1954).

[11] G.O. Gey, W.D.Coffman, and M.T. Kubicek, Cancer Res. 12, 264 (1952).

[12] J.E. Salk, Am. J. Publ. Health 47, 1 (1957).

[13] J.C.N. Westwood, J.A. Macpherson, and D.H. Titmus, Brit. J. Exp. Pathol. 38, 138 (1957).

[14] R.N. Etingof and N.D. Gabrielyan, Biokhimiya 24, 1104 (1959).*

[15] H. Tiedemann, and J. Born, Biochim. et biophys. acta 27, 656 (1958).

[16] M.I. Mostkovoi, The Practice of Processing Statistical Variations in Clinical Data [in Russian].

[17] J. Leslie and J.N. Davidson, Biochem. J. 49, XLII. (1951).

[18] J.S. Latta and D.J. Bucholz, Arch.Exp. Zellforsch. 23, 146 (1939).

[19] M. Swann, Cancer Res. 17, 727 (1957).

[20] J. Leslie, M. Jarnell, and R. Crockett, IV Internat. Congr. Biochem. (Brussels, 1958), p. 68.

[21] O. Warburg, K. Gawehn, and A.W. Geissler, Z. Naturforsch. 13b, 61 (1958).

[22] V.I. Agol, Doklady Akad. Nauk SSSR 130, 659 (1960).*

[23] V.S.Il'in, Ukr. biokh. zhur. 30, 911 (1958).

[24] V.S. Il'in, IX All-Union Conference on Physiology Biochemistry, and Pharmacology 3 [in Russian] (Minsk, 1959) p. 140.

[25] H.G. DuBuy and M.L. Hesselbach, J. Histochem. and Cytochem. 4, 363 (1956).

[26] H.G. DuBuy, Neurology 69, Suppl. 8 (1958).

[27] W.C. Schneider and V.R. Potter, J. Biol. Chem. 177, 893 (1949).

[28] J. Frederic and J. Chevremont, Arch. Biol. 63, 109 (1952).

[29] P. Hochstein, Science 125, 496 (1957).

[30] M. Woods, J. Hunter, and D. Burk, J. Nat. Cancer Inst. 16, 352 (1953).

[31] G. Ungar and S. Kadis, Nature 49, 183 (1959).

[32] S. Bessman and J. Fitzgerald, Fed. Proc. 18, 755 (1959).

[33] M.F. Gulyi, IV Internat. Congr. Biochem., (Brussels, 1958) p. 102.

THE INTERACTION OF PURINE AND PYRIMIDINE DERIVATIVES WITH FORMALDEHYDE IN AQUEOUS SOLUTIONS

M. Ya. Fel'dman

Biochemistry Laboratory, The Antipoliomyelitis Preparations Research Institute, Moscow

Translated from Biokhimiya Vol. 25, No. 3, pp. 563-569, May-June, 1960
Original article submitted September 25, 1959

The reaction of the nucleic acids with formaldehyde is of interest for two reasons; firstly, it is one of the characteristic reactions of nucleic acids from the chemical point of view and, secondly, it is a reaction which has a definite biological effect, namely, the inactivation of virus nucleoprotein [1-3]. The chemical mechanism of this reaction is of interest not only theoretically, but also in practice, as it concerns the preparation of formalin-killed virus vaccines (against poliomyelitis, encephalitis, influenza, foot and mouth disease) which is being widely applied presently.

One of the important stages in the study of the interaction of formalin with nucleic acids is the investigation of its reactions with the constituents of these compounds. Fraenkel-Conrat [1] showed that the addition of formaldehyde caused a change in the absorption spectra of the purine and pyrimidine derivatives containing an amino group, namely adenine, adenosine, and adenylic, guanylic and cytidylic acids; on the other hand, the corresponding spectra of thymine, uracil, uridine and of uridylic acid remained unaltered. The further study of these changes was hampered by the lack of methods for the investigation of the labile formaldehyde derivatives, particularly those which exist in solution, but cannot be isolated and cannot be studied under conditions where the system under investigation remains unaltered. To this type of compounds belong the various addition or condensation products formed between formaldehyde and a variety of natural products, such as amino acids, proteins and carbohydrates. It was reasonable to assume that at least some of the reactions of formaldehyde with nucleic acids and their components proceed with the intermediate, or perhaps terminal, formation of such condensation products.

The application of a method previously described [4] for the determination of free formaldehyde in the presence of its labile derivatives afforded a new approach to the solution of some of the problems concerned with the study of the interaction of formaldehyde with purine and pyrimidine derivatives.

METHODS

Materials. The synthetic samples of purines and pyrimidines (with the exception of adenine and cytosine) used in the present experiments were gifts from the Laboratory of Heterocyclic Compounds VNIKhFI. Adenine, cytosine and the nucleosides and nucleotides were commercial preparations. Adenine, adenine sulfate, adenosine, inosine, uridine, and guanylic acid were purchased from German (GFR) firms; adenylic acid was purchased from a German firm and from NB Co. and cytosine and the pentoses from NB Co. Commercial preparations of hypoxanthine (NB Co.), uracil, and thymine (Light's) were compared in experiments with the corresponding synthetic preparations obtained from VNIKhFI. Identical results were always obtained when samples of one compound, obtained from various sources, were used.

The formaldehyde solution used was a dilute formalin solution, purified by passage through activated charcoal. In a number of experiments paraformaldehyde solutions were used; the results were invariably the same as with formaldehyde.

Analytical methods. Free formaldehyde was estimated as described before [4], with some improvements.

The diffusion apparatus was prepared from lucite by turning on a lathe (this simplifies the manufacture and allows the preparation of any number of identical vessels). The inner chamber was replaced by a watch glass. The watch glass was fixed to the vessel by means of a drop of melted paraffin wax which formed an attachment on solidifying. In order to bring the test solution into the closest possible proximity with the absorbing liquid, it is necessary for the solution to fill the vessel to the upper edge of the central island without, however, running underneath the watch glass. In the apparatus of the dimensions shown in Fig. 1, 2 ml of the solution was sufficient to fill the space (as compared with 20 ml in the previously described apparatus), water (0.04 ml) was placed on the watch glass. The vessel was filled and covered with a ground glass

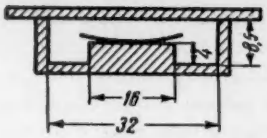


Fig. 1. Schematic drawing of the diffusion apparatus. Transverse section. Dimensions in mm.

lid sealed with a mixed paraffin-vaseline lubricant. The use of small volumes and of optimal dimensions of the diffusion vessel allowed to cut diffusion times down to 18 hours. At this time equilibrium was established and free formaldehyde concentrated on the watch glass. A sample of the solution (0.02 ml) was then removed from the watch glass and the free formaldehyde was estimated colorimetrically (with phenylhydrazine). Optical densities were measured with an SF-4 colorimeter. Nitrogen was determined by the Kjeldahl method, as modified by Conway.

Experimental details. All solutions were prepared in 1/15 M phosphate buffer, pH 7.4. Each solution contained one of the purine or pyrimidine derivatives and formaldehyde. The initial concentrations of the reactants were 0.01 M (these were checked by nitrogen analysis and iodometrically; formaldehyde was estimated in control solutions). Aliquots of the solutions were incubated in sealed tubes for 24hr at 25-26°, before the diffusion and quantitative determination of free formaldehyde. Control incubations were also carried out with 0.01 M solutions of formaldehyde alone. In the final colorimetric estimation the solution taken from the watch glass of the control vessel was used as a formaldehyde standard. Such a comparison of the total and free formaldehyde excluded several sources of error and allowed a more accurate determination of the bound formaldehyde, which was calculated as the

TABLE 1. The Interaction of Purine and Pyrimidine Derivatives With Formaldehyde in Aqueous Solutions. (initial content of reactants per test (10 ml) 100 μmoles. Final pH 7.3± 0.1; Temp., 25-26°)

Compound investigated	Formaldehyde reacted, μmoles
Pyrimidine derivatives	
Uracil	4
Thymine	2
1,3-dimethyluracil	0
Uridine	0
Cytosine	11
2-amino-6-hydroxypyrimidine (isocytosine)	5
2-aminopyrimidine	10
2,4-dihydroxy-6-aminopyrimidine *	50
Purine derivatives	
Adenine	20
Adenosine	10
Adenylic acid	9
Guanylic acid	13
Hypoxanthine	10
1,3-dimethylxanthine (theophylline)	9
2,6,8,-trichloropurine **	24
Inosine	0
1,9-dimethylisoxanthine	1
1,3,9-trimethylisoxanthine **	0
D-ribose	2
D-2-desoxyribose	0

*In view of the poor solubility of 2,4-dihydroxy-6-aminopyrimidine, it was dissolved at 50°. A crystalline precipitate appeared upon the addition of formaldehyde. The subsequent determinations with this suspension were the same as with the other solutions.

**This compound has a solubility in water somewhat below 0.01 M.

difference between the total and free formaldehyde.

Control experiments showed that, during the experimental period employed, there was no unaccounted-for disappearance of formaldehyde, nor was there any increase in the amount of bound formaldehyde after the equilibrium period chosen, even over incubation periods up to 13 days.

The chief complication in the present experiments arose from the poor solubility of some of the purine and pyrimidine derivatives. Because of this, some of the compounds (guanine, guanosine, xanthine and others) were not investigated.

RESULTS

It will be seen from Table 1 that the pyrimidines which contain an amino group differed sharply, in their reaction with formaldehyde, from uracil derivatives, which are devoid of amino groups, thus indicating the importance of this group in the reaction. In the reaction with uracil or thymine the amount of free formaldehyde which disappeared was very small and was of the same order of magnitude as the experimental error. Nevertheless, the large number of reproducible values and similar results of spectrophotometric determinations [1], indicate that this decrease is, apparently, significant. Uridine and 1,3-dimethyluracil failed to react at all with formaldehyde under the present experimental conditions, probably because of the substitution in these compounds of the mobile hydrogen in position 3.

A similar relationship held for the purine derivatives. Those containing the amino group, namely adenine, adenosine, adenylic acid and guanylic acid, reacted with formaldehyde. Additional experiments were carried out in order to check the possibility of guanylic acid reacting with formaldehyde through the hydroxyl group of the purine base (in the tautomeric form—an NH group in position 1). The most useful comparison for this purpose was that with inosine—a nucleoside with a hydroxyl in the position 6 of the purine and no amino group in position 2. Under the conditions of the experiment inosine did not react with formaldehyde, while hypoxanthine did. This interaction was apparently made possible through the mobile hydrogen atom which resonates between the nitrogen atoms in positions 7 and 9 and which is substituted by ribose in the nucleoside. The availability of position 9 as a site for formaldehyde binding was demonstrated by the case of considerable formaldehyde uptake in the reaction with 2,6,8-trichloropurine, where the only unsubstituted hydrogen is that in position 9. The attachment of formaldehyde to the -NH group in this position appears to be the only means for the reaction of 2,6,8-trichlonopurine with formaldehyde. A similar explanation holds for the reaction of formaldehyde with 1,3-dimethylxanthine since in the latter compound the mobile hydrogens in positions 1 and 3 are substituted by methyl groups, but the interaction with formaldehyde is excluded only if position 9 becomes unavailable (as in the case of 1,3,9-trimethylisoxanthine and also 1,9-dimethylisoxanthine).

Thus the hydroxypurines, containing hydroxy- (or oxo-) groups in positions 2 and 6 reacted under the conditions of the present experiments with formaldehyde not through these latter groups, but through the > NH groups of the heterocycle, in positions 9 or 7; under the present conditions only the amino group reacted with formaldehyde in adenosine and in adenylic and guanylic acids.

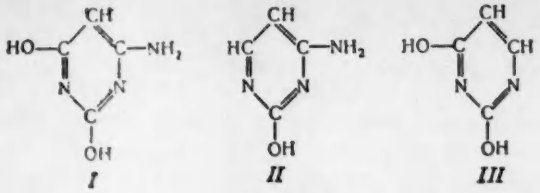
The reaction of formaldehyde with the $-NH_2$ or > NH groups also indicates the probable type of the reaction: hydrated formaldehyde reacts with such functional groups by direct condensation. In the present experiments the amount of formaldehyde bound was below theoretically possible equivalent values, which was probably due to the existence of an equilibrium with a considerable shift towards the hydrolysis of the complex formed. Some of the solutions (with adenine, adenosine, adenylic acid, hypoxanthine, thymine, uracil and cytosine) were diluted with water and subjected to distillation. The formaldehyde distilled over quantitatively.

The pronounced reversibility of the reactions, which is characteristic for the hydroxymethyl derivatives of formaldehyde, thus indicates that, of the two possible types of formaldehyde condensation products (resulting in hydroxymethyl or methylene derivatives, respectively), those encountered in the present experiment are of the hydroxymethyl type ($>NCH_2OH$).

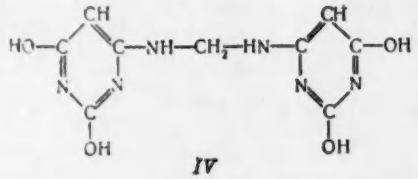
Under altered experimental conditions it was firstly desirable to investigate the effect of H^+ and OH^- ions—the most important and most generally encountered catalysts of the reactions involving formaldehyde condensations with other compounds. When formaldehyde binding by adenine, adenosine, adenylic acid, hypoxanthine and thymine was investigated in the pH range 2.4-10.8, the results were in all instances the same as observed previously at pH 7.4. Condensation was actually slightly depressed at the extreme acid or alkaline pH values. This is in accordance with the results of Staehelin [3], who found that pH variation between 5 and 8 did not affect the reaction of formaldehyde with RNA. Lauffer et al. [2] also found that the rate of inactivation of tobacco mosaic virus with formaldehyde varied little between the limits of pH 4.1-8.4. The first authors' [3] explanation of the effect, based on the absence of dissociation of the amino groups of these compounds in the pH limits studied, appears to be convincing.

The Condensation of 2,4-Dihydroxy-6-Aminopyrimidine with Formaldehyde

The compound 2,4-dihydroxy-6-aminopyrimidine (its tautomeric form is 2,4-dioxo-6-iminohexahydropyrimidine) -I, is of particular interest as a close analog of two component bases of the nucleic acids—cytosine (II) and uracil (III).



I *II* *III*



IV

In the experiment with this analog the reaction product was actually isolated. 2,4-Dihydroxy-6-aminopyrimidine was shown to react with formaldehyde quantitatively: Addition of excess formaldehyde to the supernatant caused no further precipitation and practically the entire nitrogen was removed from the solution. The stoichiometric reaction (Table 1)—one molecule of formaldehyde for two molecules of 2,4-dihydroxy-6-aminopyrimidine—corresponds to one only possible complex type, which is also in keeping with the known chemistry of formaldehyde—namely, a condensation product of the structure R-CH_2-R. The most probable site of reaction with formaldehyde is the amino group. A similar condensation product with formaldehyde is given by α-aminopyridine [5]. In the compound thus formed the methylene bridge binds the two pyrimidine bases through the α-amino groups. Such a bridge-form can also be suggested as the final product of the present reaction, particularly as a possible intermediate—the hydroxymethyl derivative—was shown to be a very probable reaction product.

The compound obtained was thus a methylene-bis derivative of 2,4-dihydroxy-6-aminopyrimidine, i.e., methylene-bis (-6-amino-2,4-dihydroxypyrimidine)—IV.

The product was a fine, crystalline powder. Its melting point was not reached at 380°. The structure was verified by analysis.

Found: 31.54% N. $C_9H_{10}O_4N_6$, calculated % N, 31.57.

Decomposition of the compound in boiling concentrated sulfuric acid with simultaneous distillation resulted in partial CH_2O recovery. When a 5×10^{-4} M solution of the compound was boiled in 27% H_2SO_4, 48.4% of the theoretically expected amount of formaldehyde was actually distilled over. The compound was not soluble in lower concentrations of acid, while in higher concentrations the losses of formaldehyde increased.

The pyrimidine component of the compound was demonstrated spectrophotometrically by its absorption spectrum (Fig. 2), which showed the characteristic peak in the ultraviolet. The solubility of the bases in sulfuric acid was considerably higher than in water, which, in 2,4-dihydroxy-6-aminopyrimidine, is due to the amino group in position 6 (cf. the solubility with that of uracil —Table 2). The practical insolubility of the methylene-bis compound in either water or sulfuric acid further suggests that the condensation involved the binding of the 6-amino group.

Only traces of methylene-bis(-6-amino-2,4-dihydroxypyrimidine) could be detected spectrophotometrically in a saturated aqueous solution of this compound. A 2.5×10^{-5} M solution, for spectrophotometry purposes, could only be obtained by dissolving it in 33% sulfuric acid. The compound was soluble in alkali and was ethanol-insoluble.

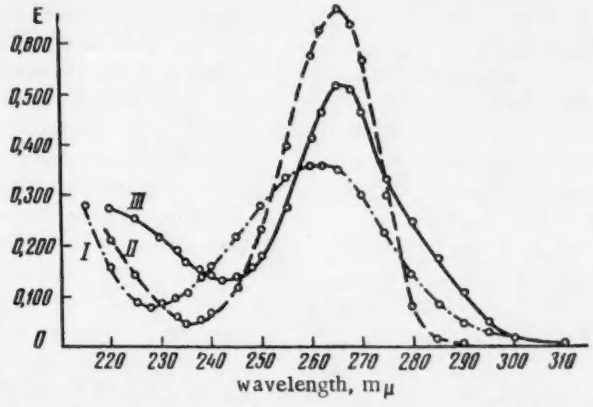


Fig. 2. The absorption spectra of uracil (I), 2,4-dihydroxy-6-aminopyrimidine (II) and of the complex (III) of (II) with formaldehyde. Molar concentrations of compounds: Uracil and 2,4-dihydroxy-6-aminopyrimidine—5×10^{-5}, the complex—2.5×10^{-5}. All extinctions were determined in 33% H_2SO_4.

TABLE 2. The Solubility of 2,4-Dihydroxy-6-aminopyrimidine and of its Methylene-bis Derivative in Water and in 1N HCl at 25° (in moles/liter)

Compound	In water	In 1 N HCl
Uracil	0.0285	0.0275
2,4-Dihydroxy-6-aminopyrimidine	0.0031	0.0866
Methylene-bis(-6-amino-2,4-dihydroxypyrimidine	0.0000	0.0000

Note. The concentration of the compound in saturated solution was determined by nitrogen analysis.

DISCUSSION

The assumption that the interaction of nucleic acids with formaldehyde results (in analogy with formaldehyde-protein interaction) in the formation of methylene bridges [3] is in accordance with the fixative and stabilizing effect of formaldehyde on biological substrates. This action, which resulted in the wide theoretical and practical application of formaldehyde in biology (morphology and immunology), is also a priori the most likely. The formation of the methylene bridges is, as a rule, always preceeded by the formation of the labile hydroxymethyl compound. In the case of nucleic acids the existence of neither type of derivative had been demonstrated.

The results of the present work demonstrate the formation of hydroxymethyl and metylene bridge derivatives (of the type R-CH_2-R) by the interaction of formaldehyde with the purine and pyrimidine bases which make up nucleic acids, or with close analogs of these bases. It is of interest that the amino groups of these bases which, in a number of their properties, are atypical behave in the reaction with formaldehyde as typical amines.

Understandably, the present results cannot be taken as direct evidence for the formation of hydroxymethyl or methylene bridge derivatives of the type R-CH_2-R in the interaction of formaldehyde with nucleic acids, but the feasibility of this reaction has been demonstrated. In this connection it is of interest to note that during the interaction of formaldehyde with nucleic acids,the functional groups that bind formaldehyde are the same amino groups [1, 3]. (The hydrogen atoms of the ring nitrogens, which can interact with formaldehyde in the case of free prrines and pyrimidines, are substituted in the nucleic acids .)

The analogy with nucleic acids in the case of 2,4-dihydroxy-6-aminopyrimidine requires special consideration. The reaction of formaldehyde with this compound resulted in the formation of a stable product of the type R-CH_2-R. The formation of a stable bond between formaldehyde and the amino groups of a synthetic cytidine polynucleotide has been demonstrated by Staehelin [3]. Thus formaldehyde reacts in aqueous solution with both 2,4-dihydroxy-6-aminopyrimidine and with the cytidine polymer by binding the same group—the 6-amino group.—with the formation, in each case, of stable, and possibly identical, bonds.

The components of nucleic acids other than purines and pyrimidines were only studied in the present work as a side issue, inasmuch as they were affected by the interaction of formaldehyde with the bases themselves. However, certain conclusions can already be made with respect to those components. Nucleosides and nucleotides reacted with formaldehyde through the amino groups of the purine and pyrimidine bases; the ribose moieties made practically no contribution to the binding of formaldehyde in aqueous solutions. Under the same condition ribose itself binds very little formaldehyde, which is in accordance with the known properties of sugars [6].

SUMMARY

A method for the determination of free formaldehyde in the presence of its complexes with a number of amino and hydroxy derivatives of purine and pyrimidine bases has been developed and used for the demonstration of sucy complex formation in aqueous solutions. The reaction was shown to involve the freely mobile hydrogens associated with the ring nitrogens in positions 9 (or 7) of the purines and, possibly, with the nitrogen in position 3 of uracil and thymine. The condensation of formaldehyde with purines and pyrimides was shown to be easily reversible. The results suggest that the labile complexes formed are hydroxymethyl derivatives.

The reaction of formaldehyde with 2,4-dihydroxy-6--aminopyrimidine resulted in the formation of a methylene-bis-amine, methylene-bis(-6-amino 2,4-dihydroxypyrimidine), which crystallized out.

The results obtained with the purine and pyrimidine derivatives lend further support to the view that the interaction between formaldehyde and nucleic acids results in the formation of hydroxymethyl derivatives and methylene bridges which link the free amino groups of the bases.

The author wishes to thank Professor A. Yu. Magidson, Dr. E.S. Golovchinskaya and Dr. K.A. Chkhikvadze who kindly provided the compounds used and afforded valuable discussion of the results.

LITERATURE CITED

[1] H. Fraenkel-Conrat, Biochim. et biophys. acta 15, 307 (1954).

[2] T.E. Cartwright, A.E. Ritchie, and M.A. Lauffer, Virology 2, 689 (1956).

[3] M. Staehelin, Biochim. et biophys. acta 29, 410 (1958).

[4] M.Ya. Fel'dman, Biokhimiya 23, 917 (1958). *

[5] A.E. Chichibabin and I.L. Knunyants, Ber. Deut. Chim. Ges. 62, 3048 (1929).

[6] G.F. Walker, Formaldehyde, (Russian translation] (Goskhimizdat, Moscow, 1957).

*Original Russian pagination. See C.B. Translation

AN APPARATUS FOR AUTOMATIC PLACING OF SPOTS ON CHROMATOGRAPHIC PAPER

M.M. Zaalishvili and F.O. Shraibman

Institute of Physiology, Academy of Sciences Georgian SSR, Tiflis

Translated from Biokhimiya Vol. 25, No. 3, pp. 570-572, May-June, 1960
Original article submitted November 19, 1959

In connection with the widespread use of the chromatographic method in biochemical investigations there has arisen a strong need for an apparatus for automatic placing of spots on chromatographic paper. Below we describe an apparatus which permits automatic placing on chromatographic paper of any number of spots simultaneously. These spots can be placed with any quantity of liquid.

Performing Mechanism

The chromatographic paper was placed in a mobile system (Fig. 1) consisting of two plastic plates 1 and 2 and fastened with two clamps 3 and 4 fixed to the lower part of plate 1. Plates 1 and 2 were connected with each other by the hinges 5, 6 whose axis was at the same time the axis of the mobile system. The mobile system was fastened by screws 7 and 8 to the brackets 10, 11 of the plastic support 12. The micropipets were placed on the platform 13 and were held in grooves by braces 14, 15 with special springs. The axis of rotation of the platform coincided with the ends of the micropipets so that when its slope was changed the position of the point of contact of the ends of the micropipets with the paper did not change. In plates 1, 2 of the mobile system, against the ends of the micropipets, there were openings 16-20 with a diameter of 20 mm for deposit and drying of the spots on the chromatographic paper. A magnet 21 was fastened to the support 12.

For the magnet we used a high-ohm electromagnetic relay with an extended armature 22 which clamped the mobile system to the micropipets. Charging of the electromagnet was controlled by the electronic scheme described below. When the magnet was shut off, the mobile system, under the influence of gravity, drew back 3 mm from the pipets. For the simultaneous and even drying of all the spots, tubes 23-26 were fixed on the support 12 against the openings 16-20; these were connected with air by plastic tubes.

Electronic Scheme

With this scheme (Fig. 2) voltage is supplied to the electromagnet (Fig. 1, 21) whose armature moves the mobile system (Fig. 1, 1 and 2) forward 3 mm. With such a position of the mobile system the micropipets are set on the horizontal platform 13 in such a way that their ends are in contact with the chromatographic

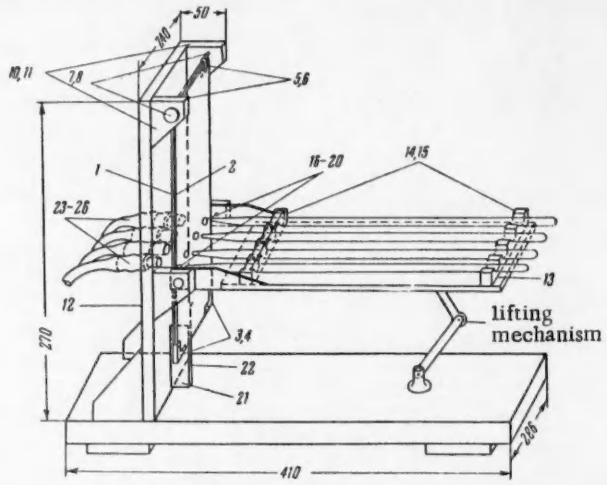


Fig. 1. Construction of the apparatus for automatic placing of the spots on chromatographic paper.

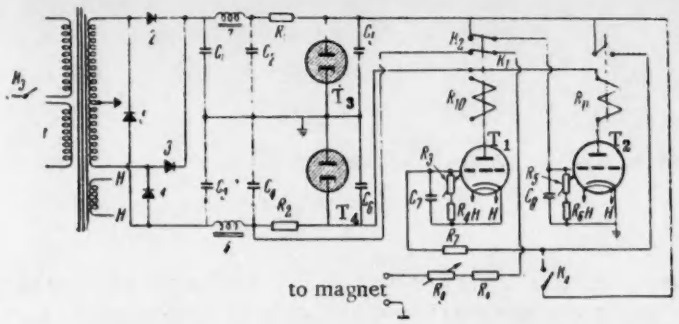


Fig. 2. Electronic scheme for control of the apparatus for automatic placing of spots. 1) Transformer LS-2; 2,3,4, and 5) DGTs-27; 6 and 7) choke coils; K_1 and K_2) contacts of relay R_{10}; K_3 and K_4) tumbler switches; C_1, C_2, C_3, and C_4) 2 microfarad (450 v); C_5) 80 microfarad (450v); C_6) 20 microfarad (450 v); C_7) 0.5 microfarad (paper); C_8) 32 microfarad (paper); R_1 and R_2) 5 kohms (wire); R_3) 1.5 μ ohms (variable); R_4 0.2 μ ohms; R_5) 4.7 μ ohms (variable); R_6) 1 μ ohm; R_7) 100 ohms; R_8) 5 kohms; R_9) 10 kohms (variable); L_1) $\frac{1}{2}$ 6N7; L_2) $\frac{1}{2}$ 6N7; L_3 and L_4) SG4.

paper but do not deform it. The tumbler switch K_4 breaks the connection which maintains the negative voltage to the grid of tube T_1, and only after this does the platform 13 with the micropipets gain the angle of slope required for emptying the liquid.

The condensor C_7 begins to discharge through the resistances R_3 and R_4. During a time-interval determined by the magnitude of C_7, R_3, and R_4, the condenser C_7 is discharged and the tube T_1 is activated; then the electromagnetic relay R_{10} included in the anode circuit of tube T_1 operates. As it operates, the relay breaks two circuits.

The first contact of the relay R_{10} switches off the mechanism which operates the electromagnet so that the chromatographic paper draws away from the micropipets. Hence C_7, R_3 and R_4 determine the time of contact of the chromatographic paper with the micropipets and also the slope of the micropipets. Since R_4 and C_7 have constant values, the time of contact is determined by varying the resistance R_3. In our scheme it is regulated in fractions of seconds up to two seconds.

The second contact, K_2 of the relay R_{10}, removes the negative charge from the grid of tube L_2. Through a time interval determined by C_8, R_5, and R_6 tube T_2 is activated and relay R_{11} supplies negative voltage to the grid of tube T_1; then T_1 is shut off, and contacts K_1 and K_2 are closed, supplying negative voltage to the grid of T_2 and voltage to the mechanism which operates the electromagnet. Hence, the values for C_8, R_5, and R_6 determine the time between the contacts of the chromatographic paper with the micropipets needed for drying the chromatographic paper. C_8 and R_6 have constant values. Therefore the time between the two contacts is determined by varying the resistance R_5. In our scheme, this time is regulated up to ten min.

The negative voltage impressed on the grid of tube T_2 shuts it off; then the contacts of relay R_{11} are broken, cutting the negative voltage from the grid of tube T_1. Thus the cycle is completed and the scheme returns to the starting position.

SUMMARY

We have suggested an apparatus for automatic placing of spots on chromatographic paper, employing an electronic scheme. The electronic scheme permits regulation of the duration of contact of the micropipets with the paper, which controls the diameter of the spots, and also the interval between placings which is needed for drying the spots.

The authors express thanks to mechanic V. Peigan who took part in constructing the apparatus.

CONGRESS TRIBUNE

Translated from Biokhimiya Vol. 25, No. 3, p. 573,
May-June, 1960

In the journal Biokhimiya No. 6, 1959, was published the first announcement of the Fifth International Biochemical Congress, which contained the conditions for the visits of foreign students at the Congress, as arranged by Intourist.

At the request of the Organizing Committee of the Congress, the management of Intourist has worked out supplementary conditions for foreign participants in the Congress.

ACCOMMODATIONS

Twenty-four rubles ($6): Two-three persons with wash stand (bath on the floor) and three single meals;

Twenty-eight rubles ($7): Two persons, with bath and three meals;

Thirty-two rubles ($8): One person with bath and three meals.

Accommodations are provided for meeting and for bus trips for group visits (groups of not less than 20-25 persons).

On arrival (by individual arrangement), the cost of meeting and local transportation will be $5 per person.

Participants in the Congress arriving from France can take the Soviet steamship of the regular line from Le Havre to Leningrad, or the railroad from Paris to Moscow. It is also possible to organize trips by special train. In this case, participants in the Congress can obtain a deduction by fulfilling the conditions of the French Railroads for special trains.

Participants in the Congress arriving from England can take any one of three Soviet steamships of the regular line from London to Leningrad or can proceed by direct train from Hook of Holland to Moscow.

Participants in the Congress who purchase the above-mentioned tours can obtain by separate fee the services of an interpreter, excursions, and autos, with payment in rubles at the existing Moscow rates. It is then possible to exchange currency at the favorable (tourist) rate, for example, ten rubles for one dollar.

Detailed information on questions connected with arrival and services for foreign students, participants in the Congress, can be obtained at tourist offices of agents of Intourist.

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Izvestiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

*BRITISH-AMERICAN TRANSLITERATION SYSTEM.

(continued)

(continued)

Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

PROCEEDINGS OF THE ALL-UNION SCIENTIFIC AND TECHNICAL CONFERENCE ON THE APPLICATION OF RADIOACTIVE ISOTOPES MOSCOW, 1957

Application of Radioactive Isotopes in Biochemistry and the Study of Animal Organisms
Jan.-Feb., 1959 heavy paper covers 20 papers, illustrated $50.00

Application of Radioactive Isotopes in the Food and Fishing Industries and in Agriculture
Jan.-Feb., 1959 heavy paper covers 16 papers, illustrated $30.00

Application of Radioactive Isotopes in Microbiology
Jan.-Feb., 1959 heavy paper covers 5 papers, illustrated $12.50

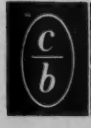
Radiobiology
Jan.-Feb., 1959 heavy paper covers 37 papers, illustrated $75.00

SPECIAL PRICE for the 4-VOLUME SET $125.00

Individual volumes may be purchased separately

The utilization of isotopes and radiation in biology, medicine, and agriculture is covered in 78 reports. Included in these significant papers are the latest Soviet techniques in the action of radiation on the living organism for the purpose of producing directed changes in plants and animals, curing of human illnesses and the utilization of isotopes as tagged atoms in the study of vital processes. Every biologist, chemist, health physicist, and physician employing the techniques should have access to this outstanding reference work.

Note: Individual reports from each volume are available at $12.50 each. We will gladly supply a detailed table of contents upon request.



CONSULTANTS BUREAU
227 WEST 17TH STREET, NEW YORK 11, N. Y.

more collected reports on...

Soviet Pharmaceutical Research

". . .a storehouse of factual references"

SOVIET research in PHARMACEUTICAL CHEMISTRY | 1956

THIS VALUABLE COLLECTION of translations not only gives researchers a more intimate insight into the thinking, methodology, and trends of the Soviet medicinal and pharmaceutical chemists, but it also reports significant contemporary advances in pharmaceutical research.

These 109 selected papers from the 1956 issues of Journal of Applied Chemistry, Bulletin of the Academy of Sciences–Division of Chemical Sciences, Journal of Analytical Chemistry, Colloid Journal, and Journal of General Chemistry will be a valuable addition to all medical and pharmaceutical libraries and should be accessible to all researchers in the field. Sections may be purchased separately as follows:

I Pharmaceutical Chemistry:
Ion Exchange
Miscellaneous
(22 papers) $12.00

II Medicinal Chemistry:
Anti-infective compounds
Steroids and related compounds
Heterocyclic compounds
Miscellaneous
(70 papers) 45.00

III Pharmacognosy:
Alkaloids
Miscellaneous
(17 papers) 10.00

THE COMPLETE COLLECTION $60.00

"At a time when the Western World eyes the achievements of science in the U.S.S.R. with a mixture of admiration and apprehension, it is gratifying to be able to read the English translation of hundreds of Russian articles in the fields of Pharmaceutical Chemistry (Vol. 1), Pharmacognosy (Vol. 2), and Medicinal Chemistry (Vol. 3)... In the application of quantitative physical measurements to problems of pharmacy, the careful recording of data and their evaluation can be termed excellent. American pharmaceutical investigators will have much to learn from these researches (Vol. 1)... Libraries of departments of medicine, chemistry, and pharmacy in universities and the pharmaceutical industry will find it essential to acquire the books, and for some specialists the pertinent volumes may become a storehouse of factual references."—*Alfred Burger in Journal of Chemical Education*

SOVIET PHARMACEUTICAL RESEARCH (1949-55)

I Pharmaceutical Chemistry:
Solubility
Stability
Ion Exchange
Emulsions
Suspensions
Gels
Miscellaneous
(74 papers) ..... $ 95.00

II Pharmacognosy:
Alkaloids
Oils
Glycosides
Miscellaneous
(87 papers) 90.00

III Medicinal Chemistry:
Structure-Activity Relationships
General
(91 papers) 100.00

THE COMPLETE COLLECTION $200.00

NOTE: Individual papers from each collection are available at $7.50 each. Tables of contents sent upon request.

CONSULTANTS BUREAU 227 West 17th Street · New York 11, N. Y.